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DIRECTION OF LOCOMOTION OF THE STARFISH (*ASTERIAS FORBESI*)¹

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I. INTRODUCTION

Jennings ('07), in his comprehensive paper on the behavior of the starfish *Asterias forreri* de Loriol, devotes considerable attention to the righting reactions of this animal. One of the results stated in this connection is as follows (loc. cit., p. 144):

There is for some reason a general tendency, seen in all the specimens, to use certain definite rays for the pulling over. A strong tendency is evident toward using the rays lying close to the madreporic

¹ For a preliminary statement of some of the results detailed in the present paper, see Cole '10.

plate. The ray *e* is used 89 times out of the 95, and the next greatest numbers are shown by the two rays lying on either side of *e*, namely *a* (56) and *d* (43). The combination of the two rays lying at the sides of the madreporic plate (*a* + *e*), was used 37 times. On the other hand, the rays lying opposite (*b* and *c*) were used but rarely, and *not once in the whole 95 experiments was the pair b + c used in combination.*

To make this statement intelligible, it will be necessary, to explain the notation used by Jennings in designating the various rays. This is shown in figure 1, which is reproduced from the diagram given by Jennings. Beginning with the arm at the

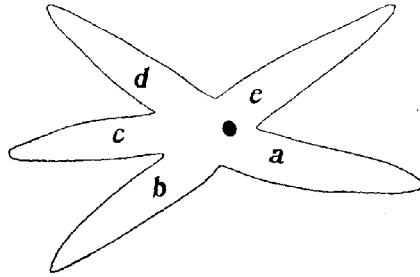


Fig. 1 Diagram of starfish, showing notation of rays with respect to position of madreporic plate (from Jennings '07). Preyer designated arms *a*, *b*, *c*, etc., as 1, 2, 3, etc. respectively (cf. p. 24). For comparison with the system adopted by Lovén, and in common use by specialists, see figure 9, p. 28.

right of the madreporite (the right hand member of the 'bivium') and going clockwise, the arms were designated arbitrarily by the letters *a* to *e* respectively. Thus it will be seen that *b*, *c*, *d*, compose the 'trivium', while *e* is the left hand member of the 'bivium.'

The question at once suggests itself: What is the reason that the rays of a certain region are used most often in the righting process? Does it mean possibly that there is something akin to an antero-posterior² differentiation? It would be inter-

² It must be borne in mind that 'anterior' and 'posterior' are here used in relation to the assumed position of the starfish in its natural locomotion, and not in their morphological sense.

esting to know in what direction, that is, with what ray forward, these starfish would have crawled if left undisturbed after righting themselves. The question then naturally occurs: Do starfishes naturally crawl with any particular region of the body (radius or interradius) forward? It is a well known fact that they *may* crawl with any part in advance. As Jennings ('07, p. 155) put it:

The starfish is not hampered by any considerations of anterior and posterior; it may move with any one of its rays in the lead, or with any interradius in advance, or indeed in any intermediate direction, so that its possibilities as to variations of direction of locomotion are really unlimited.

But, other things being equal, is this a matter of indifference? In certain echinoderms, e. g., holothurians and spatangoids, there is a well defined antero-posterior differentiation with respect to locomotion, accompanied, in most cases, by decided secondary morphological bilaterality. It was in the attempt to throw some light on these questions that the experiments reported in this paper were undertaken.

II. MATERIAL AND METHODS

While it was hoped to test the matter not only with asteroids, but with representatives of the other classes of Echinodermata as well, the only animal with which more than tentative experiments were accomplished was one of the common starfishes of the New England coast, *Asterias forbesi* (Desor).^{*} The work was begun at the United States Fisheries Laboratory³ at Woods Hole in the summer of 1909, and was continued later in the same season at the Zoological Laboratory of the Sheffield Scientific School of Yale University, at New Haven.

As it was desired to determine the direction of locomotion in the absence of external stimuli of a directive nature, it became necessary to make the conditions of the experiments as uniform

^{*} I wish to express my indebtedness to Dr. Francis B. Sumner, Director of the Laboratory, and to the authorities of the United States Bureau of Fisheries for the facilities of the laboratory.

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as possible around an axis passing through the center of the starfish and perpendicular to the surface upon which it was crawling. The presence of all external stimuli (mechanical, chemical, thermal, and light) can no more be eliminated than can those of internal origin, but so long as they are uniform over the entire animal, or bear to it the radial relation mentioned above, they need not be considered.

The earlier experiments were performed in a large circular glass dish, 36 cm. in diameter, 13 cm. deep, and having a flat bottom. At the beginning of each period of experimentation this dish was filled with fresh sea water to a depth of about 10 to 15 cm. It will be seen that at the central point on the bottom of this dish the requirements stated above regarding mechanical, chemical and thermal conditions must have been very well met. Light could, of course, have been excluded entirely, except for its desirability in enabling the experimenter to observe the behavior of the subject. Accordingly the dish was illuminated by the light from an incandescent electric bulb at the ceiling some 6 or 7 feet above, and directly over the center of the dish. Extraneous light and unsymmetrical reflection were prevented by means of a cylinder of black cloth, supported on hoops, and pendant from the ceiling, with the lamp at its center above, and enclosing the glass dish below. As a further precaution, the experiments were all performed at night, when outside the cloth cylinder was darker than within. A small peep hole permitted observation of the interior, while, by passing the hand beneath the cloth, the specimen could be manipulated.

At New Haven the experimental conditions were essentially the same, except that a glass dish of sufficient size not being available, a small pressed paper wash tub was used in its place, and the experiments being conducted in a dark-room, the cloth cylinder was dispensed with.

In the experiments made for determining simply the direction of locomotion, ten starfishes (designated Nos. 1 to 10) were used, with each of which fifty trials were recorded. Owing to the limitations in size of the receptacles employed, compara-

tively small specimens (from 5 cm. to 14.5 cm. in diameter) were chosen; but except for this, no selection was made, further than that care was taken to see that all individuals used were normally active and that all their arms were normally developed, specimens with one or more arms decidedly deficient in size,⁴ or in any way noticeably abnormal, being rejected. The method of procedure in the trials was as follows. When all was ready the starfish was picked up and held inverted with the aboral disc resting on the operator's finger tips. It was held in this position usually from a half-minute to two minutes, or until the arms of the starfish drooped down aborally. The specimen was then placed oral side down in the center of the dish of sea water, care being exercised to handle it, in so far as possible, by the disc alone, in order to avoid the possibility of an unequal stimulus which might result from handling the animal by the rays. Furthermore, that any possible unilateral stimulating effect of the environment might be eliminated, the specimen was rotated one-fifth of its circumference in each succeeding trial, the arms *a*, *b*, *c*, et cetera, being turned toward the observer successively. Between each successive trial it was removed from the water, being handled in the same way, and held inverted on the fingers for a period as described above. It was felt that if the starfish were simply moved back to the center of the dish each time, this action might not be enough to break up the action of the impulse under which it was crawling in a given direction in the previous trial. In other words, any trial might then be considered merely a continuation of the previous one. Jennings ('07, p. 141) for example, found in studying the righting reactions of *Asterias forreri* that the animals tended to right themselves in the same way as in the preceding case, that is, the impulse was retained from a previous reaction. To what extent the method employed to break up their impulse was successful in the present experiments will be discussed later (p. 16).

⁴ In the case of specimen No. 3 it was noted that arm *c* was slightly shorter, while in No. 5, arm *d* was noticeably shorter.

III. METHOD OF LOCOMOTION

Before considering in detail the direction in which the starfish crawls it may be well to consider briefly the method of locomotion. In the paper to which reference has already frequently been made Jennings ('07) does much to correct the popular conception of this process. The important point is that except on vertical or overhanging surfaces, the tube foot of the starfish acts essentially as does the leg of a higher animal, that is, "as a lever for swinging or shoving the body forward, not as a rope for hauling it forward." The sucker functions chiefly in giving the foot a firm hold and preventing its slipping. As a result of this use of the tube feet, the locomotion is not a perfectly even glide, but may often be seen to consist rather of a series of very short lunges. Furthermore, these lunges are not always exactly in the same direction but now a little to this side, now a trifle to that, resulting in a slight zig-zag movement, so slight, however, that it is apt to be overlooked without close observation.

Different starfishes show considerable individuality in their methods of progression. Certain specimens show a tendency as they crawl, even over a short course, to change the direction of locomotion, not by a turning of the body, but by a change of front, so to speak. In an undisturbed individual this change is usually very small; for example, if it starts out with ray *e* in advance, it may change more or less to the interradial area *ea*, but the arm *e* points in the same direction as before.⁵ In the experimental trials, this change of direction was usually so slight that it was inconsequential; when it was greater, the preponderating direction was recorded. In only four instances in the course of these experiments was an undisturbed crawling starfish, so long as it remained on the bottom of the dish, observed to change its course radically, or to stop and then go off in a new direction. In these four cases (specimen No. 3, trials 38 and 47; specimen No. 6, trials 12 and 16) there was no obvious

⁵ In some cases there may be a slight turning of the animal as well as this change of direction.

explanation of the departure from the normal behavior. Certain specimens showed a tendency always to change their course in the same direction, while others swayed a little first to one side and then to the other.

My observations still further confirm those of Jennings ('07, pp. 97, 115) with regard to the 'unified impulse' in locomotion. When a starfish once establishes progression in a definite direction, the tube feet of all the rays are extended with reference to the movement in that general direction, and not according to their relation to the particular rays.

As to the position of the rays during locomotion, there is again much variation; two rays may be pushed in advance,

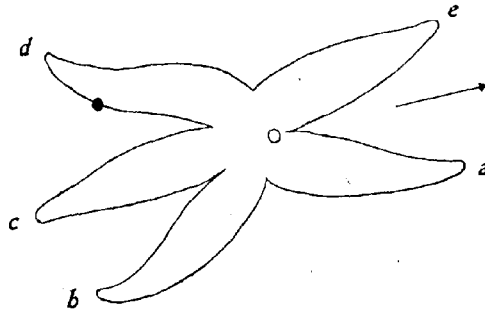


Fig. 2 Diagram of starfish crawling with two rays in advance and three following. Arrow indicates direction of locomotion.

with three following (fig. 2) or three may be in advance and two behind (fig. 3). Sometimes the arms are all flattened to the surface on which the starfish is crawling; at other times, or in other individuals, they are curled upward at the tips. In certain instances it was noted that flexible specimens were more active than the more rigid ones, and such specimens would go through fifty successive experimental trials often with no appreciable slowing in the rate of locomotion. The more sluggish individuals sometimes slowed down towards the end of the series, and in one case (No. 5) it was necessary to fill out the last five trials of the fifty on the succeeding day.

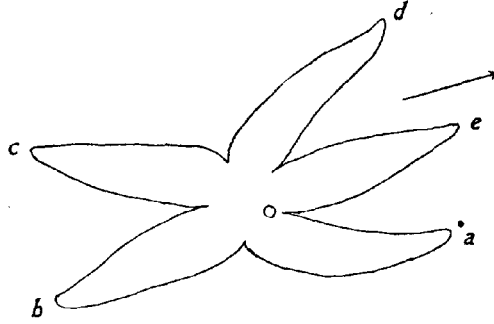


Fig. 3 Diagram of starfish crawling with three rays in advance

IV. EXPERIMENTAL RESULTS

1. *Direction of locomotion*

In recording the individual trials in the experiments note was made as to which arm was directed toward the observer at the beginning of the trial (i.e., the orientation of the specimen) and the part of its own body which was in advance as it crawled. Since the orientation was changed in the successive trials, the actual direction of movement was of course different if the animal crawled with the same ray in advance. The orientation at the beginning of the trial was recorded that it might serve as a check on the uniformity of the environment; but since the position of original orientation showed no effect in the results of the experiments, it will not be further considered, and we need concern ourselves only with the direction of advance with respect to morphological relations. This was recorded only for radii and interradii. There were bound of course to be some cases of doubt; but these were comparatively few, and an error here would be of little consequence, since it would merely throw the reading to the next radius or interradius to right or left, and these would tend to balance each other. That such was actually the case is indicated by the evenness of the totals for the different adjacent positions as shown in table 1, which presents

a summary of the results of the whole set of experiments. In the horizontal rows are recorded the results of trials with each of the ten individuals. The vertical columns show the number of trials in which the respective radii and interradii were in advance. Inspection of the totals shows that on the whole there was a considerably larger number of trials in which arm *c* was in advance than any other arm or interradius. It will be noticed, furthermore, that there is in general a falling off as one goes in either direction from this position. This is shown more clearly

TABLE 1
Distribution of crawling trials

INDIVIDUAL	RADIUS OR INTERRADIUS IN ADVANCE										TOTAL
	<i>c</i>	<i>cd</i>	<i>d</i>	<i>de</i>	<i>e</i>	<i>ea</i>	<i>a</i>	<i>ab</i>	<i>b</i>	<i>bc</i>	
1	2	6	7	8	14	5	3	1	2	2	50
2	5	5	8	2	10	4	4	1	7	4	50
3	2	5	13	1	2	6	5	5	7	4	50
4	5	12	3	4	13	1	0	3	2	7	50
5	5	0	1	1	16	13	5	1	3	5	50
6	4	2	4	3	11	9	4	5	5	3	50
7	1	0	3	7	5	6	11	9	3	5	50
8	0	1	8	9	7	7	9	4	4	1	50
9	1	5	8	6	4	4	6	4	6	6	50
10	8	7	9	2	3	6	8	2	1	3	49 ¹
Total....	33	43	64	43	85	61	55	35	40	40	499

¹ Through oversight only 49 trials were made with specimen No. 10.

in figure 4, where the totals of table 1 are shown plotted in the dotted line. In this figure the abscissal divisions represent the radii and interradii from left to right, while each of the ordinal spaces represents ten trials.

If we consider all the movement in relation to the radii alone, the preponderating direction of movement stands out somewhat more clearly. This may be done legitimately by apportioning to each of the radii, in addition to its own records, one-half of those for each of the adjacent interradii to right and left. The result of such a redistribution is shown in table 2. It is here easily seen that the preponderating direction in which starfish No. 1 crawled was with arm *c* in advance. The same is true

TABLE 2
Crawling trials apportioned to radii

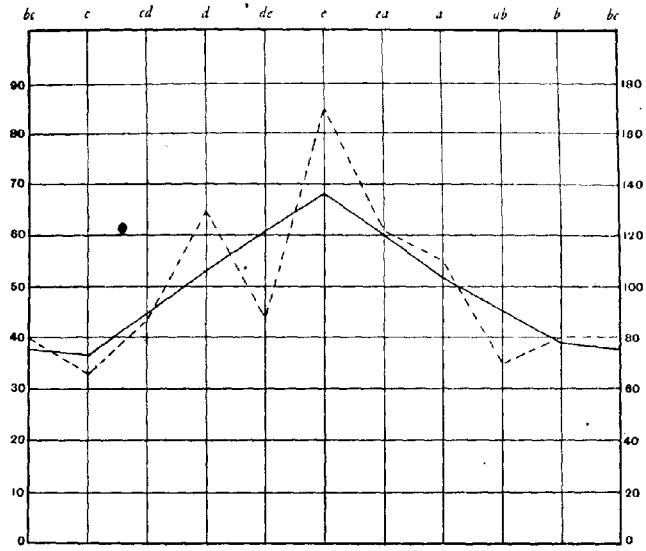
INDIVIDUAL	RADII					TOTALS	PREPONDERATING DIRECTION
	<i>c</i>	<i>d</i>	<i>e</i>	<i>a</i>	<i>b</i>		
1	6.0	14.0	20.5	6.0	3.0	50	<i>e</i>
2	9.5	11.5	13.0	6.5	9.5	50	<i>e</i>
3	6.5	16.0	5.5	10.5	11.5	50	<i>d</i>
4	14.5	11.0	15.5	2.0	7.0	50	<i>e</i>
5	7.5	1.5	23.0	12.0	6.0	50	<i>e</i>
6	6.5	6.5	17.0	11.0	9.0	50	<i>e</i>
7	3.5	6.5	11.5	18.5	10.0	50	<i>a</i>
8	1.0	13.0	15.0	14.5	6.5	50	<i>e</i>
9	6.5	13.5	9.0	10.0	11.0	50	<i>d</i>
10	13.0	13.5	7.0	12.0	3.5	49	<i>d</i>
Total	74.5	107.0	137.0	103.0	77.5	499.0	

for Specimen 2; for No. 3 it is arm *d*; and so forth, as indicated in the last column to the right in the table. This column shows that in six of the ten individuals used, the preponderating direction of crawling was toward arm *e*; in three it was toward arm *d*; while in one arm *a* was most often in advance. In no case was the preponderating movement toward either *b* or *c*. This tendency to move in the direction of arm *e* is plainly indicated when the records for the ten individuals are added, as shown in the bottom row of the table. The same fact is represented graphically by the solid line in figure 4, where these totals are plotted; the ordinal spaces in this case being given a valuation of twenty trials in order to make the two curves comparable. The 'mode,' as before, is clearly at *e*; but in this case the 'curve' drops off smoothly and with remarkable symmetry on the two sides.

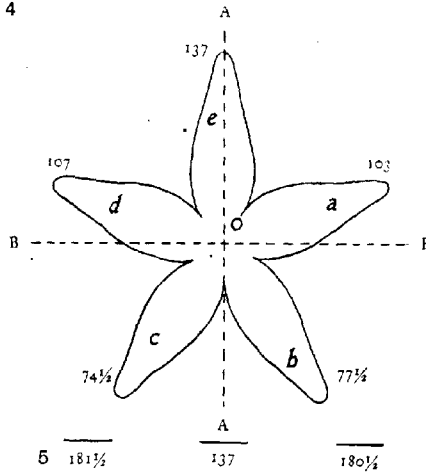
In figure 5 these valuations are referred to a diagram of a starfish, where the symmetry of the 'curve' in figure 4 shows

Fig. 4 Plotting of number of times each radius and interradius was in advance in the crawling experiments. Dotted line from data given in table 1; solid line from data of table 2.

Fig. 5 Diagram of starfish, showing the number of trials accredited to each arm as 'director' (cf. fig. 4 and table 2). *AA*, line of bilateral symmetry with respect to the records; an approximately equal number of trials fall to each side. The line *BB* has 347 records 'ahead' of it and only 152 'behind.'



4



an interesting relationship to the animal, for a line *AA* drawn through arm *e* and the interradian area *bc* divides the crawling records almost equally into halves; 137 of the 499 records lie on the line, while of the remaining 362 trials, 181.5 are on the left and 180.5 on the right. Or if a line *BB*, at right angles to *AA* be drawn through the center of the disc, 347 records are in a direction 'ahead' of this line, while only 152 are 'behind' it.⁶

From the foregoing it may be concluded that, *although the starfish Asterias forbesi may move with any ray in advance, in the absence of directive stimuli, as shown by a large number of trials, it was most often the one lying next to the left of the madreporic plate which went ahead.* Using direction of movement as a criterion, this may then perhaps be considered the 'physiological anterior' of the animal.

If we turn again now to the experiments of Jennings, it will be recalled that in his study of the righting reactions he found the ray *e* used most often, namely 89 times out of 95, "and the next greatest numbers [were] shown by the two rays lying on either side of *e*, namely *a* (56) and *d* (43)." His results are thus directly comparable to those on the direction of movement, and in each case a greater activity of the ray *e* and the rays on either side of it is probably indicated. A somewhat similar determination of direction of movement dependent upon a differential activity of the organs of locomotion has been demonstrated by the writer (Cole '01) in the pycnogonid, *Anoplodactylus lentus*, in which the legs assume an essentially radial position.

2. Relation of direction of locomotion to length of arm

A possible explanation of the greater activity of the 'anterior' rays (if we may so call them) is suggested by the observation mentioned in the footnote on page 5 that in some of the specimens used the arms *b* and *c* (the 'posterior' arms) were noticeably shorter. Unfortunately the actual specimens used in the

⁶ If the rays had been used indifferently, approximately 100 records would be expected for each ray.

experiments were not accurately measured nor preserved, but in order to ascertain whether perhaps this condition of shorter 'posterior' arms held generally, though in a less striking degree (since otherwise it would have been noted) careful measurements of the length of arm have been made on a series of 116 specimens of *Asterias forbesi* from the Woods Hole region, and of about the size of those used in the experiments. A brief statement of the results of these measurements will suffice for the present discussion. The specimens measured were a selected sample only in that individuals about the size of those used in the experiments were included and that those having arms of obviously disproportionate lengths (probably regenerating arms) were excluded. This was done also in selecting individuals for the experiments. Nevertheless, in measuring this somewhat restricted lot, one was most impressed by the considerable variation in arm length and its apparent irregularity as to position. It was quickly discernible that no one arm, nor pair of arms, was regularly the longest or the shortest. The number of times each arm occurred as the longest or as one of the two longest (in cases where the longest two measured the same) and as the shortest or one of the two shortest, was as follows:

ARM	NUMBER OF TIMES				
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
Longest or one of two longest . .	53	41	25	22	36
Shortest or one of two shortest . .	30	28	40	41	31

These figures correspond in a general way to the results on locomotion, and in so far would appear to show a correlation between length of arm and the frequency with which it moves in advance. Thus it will be observed that arms *c* and *d* are longest the least number of times, while arm *a* is most often longest (or one of the two longest). Conversely arms *c* and *d* are the shortest more often than the others, and arms *e* and *a* are shortest the least often. This may be seen most readily where the figures are plotted as in figure 6, which should be compared with figure 4. It will be noted that except for the

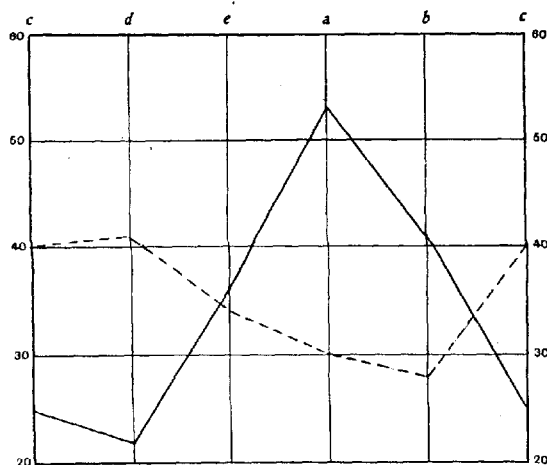


Fig. 6 Plotting of number of times in 116 specimens in which a particular arm occurred as the longest or one of the two longest (solid line) or as the shortest or one of the two shortest (dotted line).

fact that the mode falls at *a* instead of *e*, the curve for the 'longest' arm, represented by the unbroken line in figure 6, corresponds closely to the curve for direction of locomotion in figure 4, while in a general way the curve for the 'shortest' arm (dotted line) is the converse of these.

If now we consider the mean lengths of the respective arms of the 116 specimens, although the differences are very small, and perhaps insignificant, nevertheless we find that their distribution corresponds to that just given, namely, *e* and *a* have the greatest mean length, *c* and *d* the shortest. The exact figures are:

ARM	<i>c</i>	<i>d</i>	<i>e</i>	<i>a</i>	<i>b</i>
Mean length in millimeters ⁷	46.30	46.23	46.91	46.93	46.74

⁷ Later computation of the probable error of the differences between the mean lengths of the respective arms as here determined makes the value of these differences extremely doubtful. The striking fact is that, considering the often very

As noted above, however, the axis which marks the approach to bilaterality in these determinations (A.A, fig. 7) does not accord exactly with that with respect to direction of locomotion (A.A, fig. 5); for whereas in that case it passed through arm *e* and between arms *b* and *c*, here it obviously passes through arm *a* and the interradius *cd*.

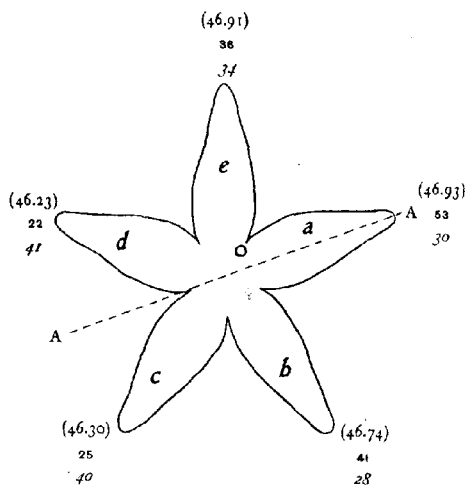


Fig. 7 Diagram of starfish, showing number of individuals in 116 specimens in which a particular arm was longest or one of the two longest (black face figures) and in which it was shortest or one of the two shortest (italic figures). Compare with figure 6. Figures in parentheses are the mean lengths in millimeters of the respective arms of the 116 specimens as determined. A.A, line of symmetry with respect to these figures.

Considering the considerable amount but irregular distribution of the variation in arm length and the very small difference in mean length of the different arms, as well as the difference

marked difference in arm length of a given individual, the mean arm lengths of the 116 specimens should be practically equal. The greater individual variation gives somewhat greater value to the tabulation on p. 13 of the number of times a particular arm occurred as the longest or shortest than to the mean lengths. But even if these figures could be relied upon the fact would still need to be considered that they do not refer to the actual specimens used in the experiments.

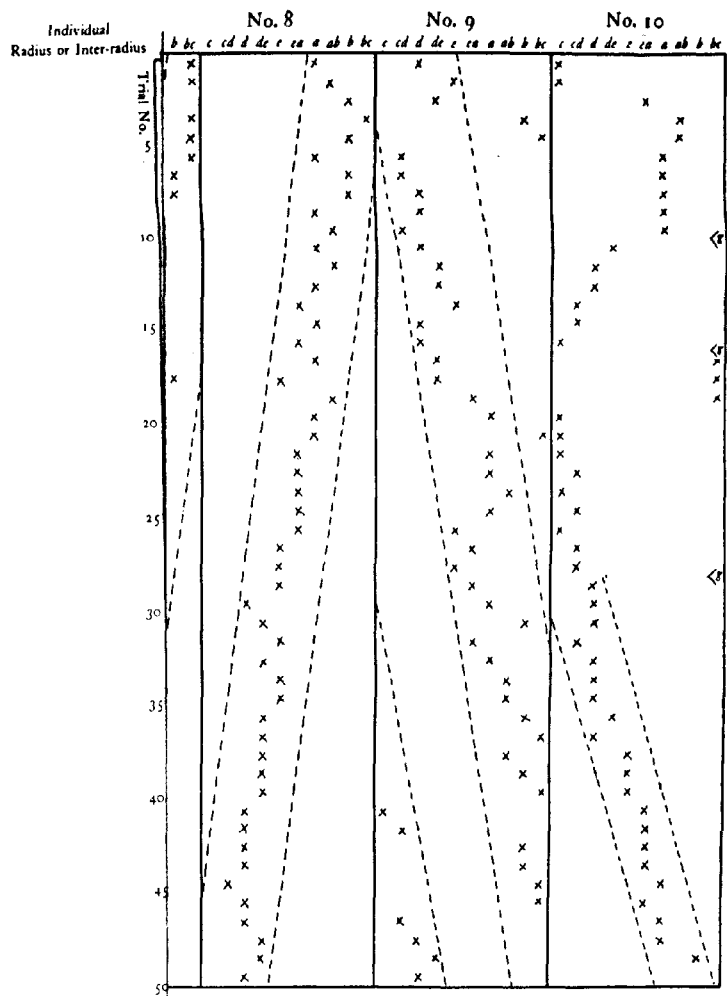
in the axes of bilaterality, it seems very doubtful whether the tendency to bilaterality in this respect can have significance in accounting for the noticeable bilaterality with respect to locomotion, in spite of the fact that the evidence seems to show that on the whole there is a slight tendency for arms *e* and *a* to be longer than the others. One is inclined to believe rather that both these results may be a more or less complete expression of factors or tendencies of development or organization.

3. *Persistence of the impulse*

Reference has already been made (p. 5) to the 'impulse' of a starfish to move in a certain direction, once it has established movement in that direction—a sort of momentum of physiological reaction which causes a certain behavior to persist for a time even against an adverse stimulus (Jennings '07, p. 115). This fact has more recently been confirmed by Cowles ('11, p. 103), who remarks:

A characteristic of the starfish is that when once the impulse to move in a certain direction is formed, the starfish is quite persistent in its behavior and continues to move in that direction; so when the creature reaches the wall it ascends owing to the persistence of the impulse

This inherent stubbornness of *Asterias forbesi* may be seen in table 3, which displays in detail the results of the whole series of trials made in the present experiments, in the order of their sequence. Here it is evident that the precaution taken to break up the locomotive impulse (cf. p. 5) was inefficient, for although there is some variation in the part advanced in successive trials, in general the same one remains the temporary 'anterior' for a considerable number of trials. Specimen No. 4 may be taken as an example. In this case the subject first crawled with the interradial area *ca* as 'anterior,' then followed seven trials with *e* in advance, next one with *de*, one with *d*, another with *de*, three with *e*, again *de*, three more *e*, and so on. The degree of this persistence is shown by the records better than it can be described.



slowly and after trial 41 refused to crawl any more; trials 45-50 made
 angled to e.
 then changed to e.
 of righting reaction.

4. *Rotation of the impulse*

Inspection of table 3 reveals another interesting relation existing in the trial records. This is what may be called a tendency to 'rotation of the impulse.' Not only is there a slight vacillation in the direction of the impulse, but there appears to be a well-marked tendency for the impulse as a whole to shift gradually around one way or the other. This was more pronounced in some individuals than in others. It is especially marked in the records of specimens No. 4, No. 5, No. 7, No. 8 and No. 9. With the records spread out flat as they are here the records of successive trials tend to trail out diagonally across the space allotted to the individual, as is indicated by the diagonal dotted lines enclosing them. But to express the proper relationships they should be plotted on a cylinder (for on the animal *c* and *bc* are adjoining) and the tendency of the impulse to rotate in one direction or the other would then be expressed by the spiral path of the records around the cylinder, to right or left as the case might be. This would bring the figures lying on each side of the flat diagrams together and make the result appear more striking.

In the case of five of the starfishes (Nos. 1, 3, 6, 9 and 10) the direction of rotation of the impulse around the animal is plainly to the right, or clockwise; in four (Nos. 4, 5, 7 and 8) it is as evidently to the left, or counter clockwise. In only one specimen (No. 2) is there definitely shown a change in the direction of this rotation during the recording of the fifty trials. This individual was apparently right-handed in this respect at first, and changed to left-handed after about the fifteenth trial. There is nothing recorded in the notes which would appear to furnish an explanation of this change.

It will be observed that the width of the 'paths' between the dotted lines in the table is in a degree a measure of the 'intensity,' if we may so call it, of the impulse; in some cases the impulse remained so nearly fixed in a particular region that trial after trial the starfish crawled with the same ray forward. Take for example, specimens Nos. 3, 4, 5, 7 and 8 (after about trial

19). On the other hand, specimen No. 6 has a broad 'path,' indicating that although the impulse was directed toward the same general region it vacillated considerably from side to side. It was noted that specimens Nos. 4 and 5 were relatively stiff or rigid and comparatively inactive, while No. 6 was flexible and moved at a good rate throughout the fifty trials. This fact may or may not be of significance.

The slant of the 'path' is similarly a measure of the 'rotation' of the impulse as a whole. This was not correlated in any definite way with the condition of the starfish, so far as could be observed, but during the course of the experiments the impression was gained that an individual was often comparatively inactive during the first few trials—that it had to be put over the course a few times before it became 'waked up to the work,' so to speak—and in a number of cases at least it slowed down toward the end of the experiments as if becoming fatigued* (for example, see No. 5, which refused to crawl after the forty-fourth trial). In this connection it may be pointed out that a considerable number of the cases (Nos. 2, 5, 6, 7, 8, 9 and 10) seem to show a tendency to start at the sides of the diagrams (in the regions or arms *b* and *c*), to cross the space diagonally, and to end again at the opposite side below. If the impression stated above is true, then this means that in general when the experiments were started with the various specimens they were comparatively inactive and tended to crawl 'backward' (if we admit that a physiological 'anterior' has been established), i.e., with the general region *bc* in advance; gradually the impulse swings around until they are crawling 'forward,' and finally as they become fatigued, it moves on around to the original position. This matter should have been tested by continuing the trials much further, but it was not realized at the time, and there appeared no obvious reason why fifty trials with each individual were not sufficient.

Whether or not the above relations prove to be general, nevertheless the records do seem to establish beyond a doubt the

* Glaser ('07, p. 206) mentions changes from active to sluggish behavior and the reverse, in *Ophiura*, for which there was no obvious explanation.

gradual rotation of the impulse. In seeking an explanation for this two possible ones suggest themselves, of which one is physiological in the strict sense, the other psychological, or based on behavior. According to the first of these, the change in direction may result from fatigue (whether of muscles or controlling centers) which results from activities being continuously directed one way, and as a result they shift to a new position, somewhat as the control of pulsation in a medusa passes from one center to another around the periphery. On the other view, this would probably be a type of modifiability of behavior analogous to that of Stentor and other lower organisms, which, as Jennings ('06, chapter 10) and others have pointed out, change their mode of reaction when one mode has been tried for a time and proves ineffectual. In this case the impulse in any one direction is ineffectual for the starfish because it fails to 'get anywhere,' and the impulse accordingly shifts to a new position, just as Stentor may bend first one way and then another to avoid an unfavorable stimulus. This 'regular' rotation of the impulse would probably not take place under any except uniform conditions; if the starfish were to come to an uneven surface or to food, these new stimuli would undoubtedly influence the further direction of the impulse. Like most cases of modifiability of behavior, this behavior of the starfish may be adaptive in nature—at least it would prevent its travelling continually in a straight line.⁹ Indeed, if we could imagine a starfish on a stretch of level, smooth sea-bottom where directive stimuli were absent, it would, according to these results, tend to swing around in a large circle and come to rest near where it started out.

5. Relation of arms used in righting to direction of locomotion

In table 4 are shown the results of a number of tests to determine what relation exists between the arms used in righting when the starfish is placed on its aboral surface and the direction of locomotion previous to and subsequent to the righting reaction. The data may be summarized as follows:

⁹ As has been mentioned (p. 6) the straight course is also frequently swerved from somewhat, even though the same ray remains in the lead.

ARMS	<i>c</i>	<i>cd</i>	<i>d</i>	<i>de</i>	<i>e</i>	<i>ea</i>	<i>a</i>	<i>ab</i>	<i>b</i>	<i>bc</i>
Crawling previous to test.....	2	6	5	1			3		2	
Arm or arms used in righting.....		3			2	16		1		2
Crawled subsequent to righting.....	2	9	5	1			2			3

This shows that whereas the four specimens used in these tests righted themselves on arms *ea* sixteen out of twenty-four times, previous to the trials they had been in nearly all cases crawling in a direction nearly opposed to these arms, and moreover, they continued locomotion in the same general direction after righting themselves. An examination of the individual records reveals the same relations in the great majority of cases:

TABLE 4

Relation of arms used in righting to direction of previous and subsequent crawling

INDIVIDUAL	PREVIOUSLY CRAWLING	ARMS USED IN RIGHTING	SUBSEQUENTLY CRAWLED
No. 9—after trial 50.....	<i>d</i>	<i>ea</i>	
No. 10—before trial 1.....		<i>ea</i>	<i>c</i>
No. 10—after trial 10.....	<i>a</i>	<i>e(b)</i>	<i>cd</i>
No. 10—after trial 16.....	<i>c</i>	<i>e(ab)</i>	<i>bc</i>
No. 10—after trial 28.....	<i>cd</i>	<i>ea</i>	<i>cd</i>
No. 10—following day.....		<i>cd</i>	<i>bc</i>
No. 10—following day.....	<i>bc</i>	<i>bc</i>	<i>a</i>
No. 10—following day.....	<i>a</i>	<i>cd</i>	<i>d</i>
No. 12—trial 1.....		<i>bc</i>	<i>a</i>
No. 12—trial 2.....	<i>a</i>	<i>ea</i>	<i>de</i>
No. 12—trial 3.....	<i>de</i>	<i>ea</i>	<i>cd</i>
No. 12—trial 4.....	<i>cd</i>	<i>ab</i>	<i>c</i>
No. 12—trial 5.....	<i>c</i>	<i>ea</i>	<i>cd</i>
No. 14—trial 1.....		<i>ea</i>	<i>d</i>
No. 14—trial 2.....	<i>d</i>	<i>ea</i>	<i>d</i>
No. 14—trial 3.....	<i>d</i>	<i>ea</i>	<i>cd</i>
No. 14—trial 4.....	<i>cd</i>	<i>ea</i>	<i>cd</i>
No. 14—trial 5.....	<i>cd</i>	<i>ea</i>	<i>cd</i>
No. 14—trial 6.....	<i>cd</i>	<i>ea</i>	<i>d</i>
No. 14—trial 7.....	<i>d</i>	<i>ea</i>	<i>d</i>
No. 14—trial 8.....	<i>d</i>	<i>ea</i>	<i>cd</i>
No. 14—trial 9.....	<i>cd</i>	<i>cd</i>	— ¹
No. 14—trial 10.....		<i>ea</i>	<i>bc</i>
No. 14—trial 11.....	<i>bc</i>	<i>ea</i>	<i>cd</i>

¹ Remained quiet 12 minutes after righting itself.

The subsequent locomotion in a direction opposite to the arms which have been used in righting is easier to understand in many cases than why the starfish should use those rays in turning over. But even here there is difficulty in reconciling the direction of the co-ordination in righting with that of the subsequent locomotion. Let us suppose for example a starfish placed on its 'back,' that is, oral side up. Let us suppose, furthermore, that it has already succeeded in twisting rays *c* and *a* and attaching them to the substratum, and that a co-ordinated impulse toward righting on these arms has become established. The tube feet of all the rays would accordingly be extended in that direction, as indicated in the accompanying diagram (fig. 8 a). In some cases these two rays will swing all the others over freely, in which case it would be expected that the impulse would be still towards *ac*, and that the animal would crawl in that direction. It much more frequently happens, however, that one or more of the other arms become attached before the animal is completely righted. Thus in figure 8 b, *e* and *a* are represented as still attached; *e* has swung over and attached at its tip between *e* and *a*; *d* has twisted and attached before crossing over, and *b* is free. Ray *c* now becomes the dominating factor in pulling the other arms over. To do this its tube feet must action by pulling toward the tip of the ray, whereas previous to its attachment they were directed towards its base (cf. fig. 8 a). Arm *b*, being free, is now pulled over into position between *a* and *c*; but as *d* is attached it remains crossed over *e*, even after the starfish is completely righted. It is now, however, swung over, with the help of the movement of the whole animal *c*-ward, and locomotion now continues in that direction. It will thus be seen that between the time the creature is in the position shown in figure 8 a, and when it is completely over and moving in a general direction opposite to that of the rays on which it turned, there must be a reversal in the direction of the co-ordinated impulse of the tube feet. The details of this were not studied as carefully as they should have been owing to the fact that the data on the righting and locomotion were not tabulated until there was no opportunity to continue the experiments.

No simple explanation appears to offer itself as to why the converse of the above should be true, namely that a specimen which had been crawling *c*-ward should, when placed on its

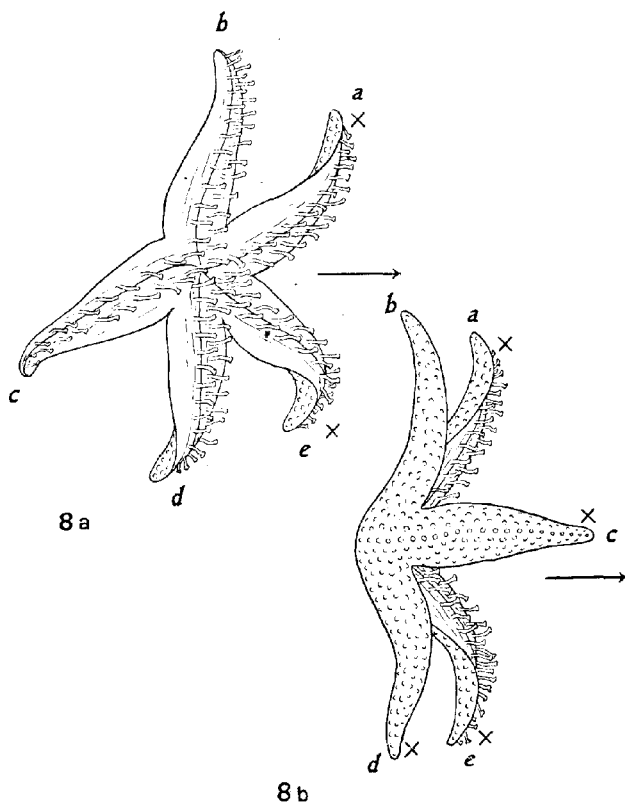


Fig. 8 Diagrams of starfish righting itself. Arrow indicates direction of turning; note that all tube feet are extended in that direction. Arms attached to substratum are indicated by X.

aboral surface, use more often the rays opposite (*e* and *a*) in righting itself. This is a point deserving further study.

At best the results in this connection seem rather paradoxical. Jennings found that his starfishes used ray *e* more often than

any other in righting themselves; in the present experiments it was found that *Asterias forbesi* moves more often with ray *e* in advance than with any other. Furthermore it was found in the comparatively few experiments which were made with respect to righting that the East Coast starfish also used rays *e* and *a* most frequently for this purpose, but *these individuals, in the great majority of cases, then crawled with the opposite rays in advance.* Which way would Jennings' starfish have crawled?

V. COMPARISON WITH OTHER ECHINODERMS

It would be interesting to know whether the tendency for a predominance of movement to be with a particular region of the body forward obtains in other starfishes and in the other groups of (practically) radial echinoderms. The statement is not uncommonly made categorically by authors, based only on general observations, that no preference is shown for a particular ray or region. Thus Cowles states in a preliminary report ('09, p. 128): "Echinaster in locomotion does not show any tendency to use a special ray, or pair of rays, as directors." He admits later, however, that although this conclusion was "based on a considerable number of observations with directive light excluded," it was "not a careful statistical study" (Cowles '11, p. 98). Grave ('00, p. 86) remarks similarly: "No preference as to which arm should precede could be found in an adult ophiuran, each arm being equally capable of going before, making the stroke, or following behind." But while each arm might be 'equally capable' of performing these actions, it does not follow that they would be used equally for that purpose. Bohn ('08, p. 29) says regarding the relative value of the arms in *Asterias rubens* that commonly they are the same functionally, but if any are shorter or are mutilated they have a relatively smaller value. Further on in the same paper (p. 43) he asserts that the small specimens of this species (rays 1 cm. in length), which are found on the *Zostrea*, and which are very active and in general show a positive phototropism, use all their arms with practical indifference. Larger specimens, however, sometimes

appear to evince "a sort of preference for certain arms;" nevertheless the general conclusion appears to be that no ray has especial value as a director. This, like many of Bohn's conclusions, one wishes might be based on a more extended series of observations.

E. C. and A. Agassiz ('65) state that "Cribrella [Cribrella oculata = Henricia sanguinolenta] moves usually with two of the arms turned backward, and the three others advanced together, the two posterior ones being sometimes brought so close to each other as to touch for their whole length." They make no statement, however, as to whether it is usually the same arms which take these positions.

1. Preyer's experiments on starfishes

Preyer ('86-7) appears to be the only person who has previously investigated this question in anything like a statistical way, and although he concluded negatively as to the preferential use of any particular arm or arms as directors, his results are worthy of examination in some detail. His method of experimentation was careful and ingenious, and one feels inclined to place confidence in what he speaks of as these 'sehr zeitraubenden Versuchsreihen.' He laid the animals on the top of a glass support, which was hemispherical above, and so arranged that while the greater parts of the rays of the specimen were immersed in the water, their basal part and the central disc were exposed to the air. The support was filled with water except for an air bubble at its top, by means of which it could be accurately leveled. Finally the specimens were oriented with a given ray to the north, east, south and west in successive trials, in order to neutralize the possible interference of any inequalities in the surroundings. The starfishes and brittle stars used naturally tended to crawl down one side of the glass support so as to immerse themselves completely in the water, and Preyer recorded what ray was in advance in this movement. He numbered the rays 1, 2, 3, 4 and 5, beginning with the one which has the madreporite at the left of its base and counting around clockwise, these numbers therefore corresponding respectively to rays a ,

b, *c*, *d*, and *e*, as denominated by Jennings. In case two adjacent rays took an equal part in the lead, 'one-half time' was accredited to each.¹⁰ Except for *Luidia*, which is a seven-rayed form, Preyer's ('86-7, p. 218) results are brought together in table 5.

Preyer states that the brittle-stars *Ophiomyxa* and *Ophioderma* showed just as little preference for any particular ray; but a careful inspection of table 5 will show that this conclusion regarding the starfishes was scarcely justified by the results presented. In fact these results show a striking similarity to

TABLE 5
Summary of Preyer's results on the direction of locomotion

SPECIES	NUMBER OF INDIVIDUALS TESTED	NUMBER OF TIMES EACH ARM WAS USED AS DIRECTOR					TOTAL	NUMBER OF TIMES EXPECTED IF USE WERE INDIFFERENT
		<i>a</i> (1)	<i>b</i> (2)	<i>c</i> (3)	<i>d</i> (4)	<i>e</i> (5)		
<i>Astropecten bispinosus</i>	6	16.5	14.5	4.0	9.5	9.5	54.0	10 to 11
<i>Astropecten pentacanthus</i>	4	7.5	3.5	7.0	14.0	18.0	50.0	10
<i>Astropecten aurantiacus</i>	3	18.5	8.0	9.0	12.5	8.0	56.0	11 to 12
<i>Asterias glacialis</i>	1	5.0	4.5	5.0	4.0	5.5	24.0	4 to 5
Total	14	47.5	30.5	25.0	40.0	41.0	184.0	36 to 37

those presented in the earlier part of this paper. Not only is this apparent in the individual records for each species (note the small number of times *c* was used as compared with the ray opposite it), but is especially noticeable when comparison is made of the totals (cf. table 5 with table 2, p. 10). In the case of Preyer's results, however, the plane of bilaterality would not pass through the radius *e*; but the plane which would have most nearly an equal number of records on each side, would cut through the interradius *ea* and ray *c*. Such a plane has eighty-one records to the left, seventy-eight to the right. So if we thus lump together then the four species of starfishes with

¹⁰ This accords with the treatment of the data in the present paper (cf. table 1, and fig. 4).

which Preyer worked (which seems permissible considering the essential similarity of the records) the 'physiological anterior,' as determined by the direction of locomotion, is the interradius *ea*.

Finally, if we combine Preyer's results with those obtained on *Asterias forbesi*, making a total of 683 trials with five different species of starfish, the result is as follows:

		RAY			
		<i>e</i>		<i>a</i>	<i>b</i>
Preyer's results.....	25.0	40.0	41.0	47.5	30.5
<i>Asterias forbesi</i>	74.5	107.0	137.0	103.0	77.5
Total.....	99.5	147.0	178.0	150.5	108.0

Here it will be seen that the plane of bilaterality passes through *e*, which is by this token the 'physiological anterior.'

2. Echinoids and other echinoderms

Aside from the few experiments of Preyer on ophiurans, and which he mentions only in a general way as giving negative results (see p. 25), no effort appears to have been made to determine definitely whether preference is given to a particular ray or radius in the other groups of echinoderms outside the asteroids. Grave has already been quoted (p. 23) as saying that in *Ophiura* 'each arm is equally capable' of acting as director, and this appears to be the general impression.

What has just been said of the ophiurans may apparently be applied equally well to the radial sea-urchins—that is, that they may crawl with any part of their circumference in advance. There are, however, certain echinoids which have assumed a secondary bilateral symmetry, and are accordingly especially adapted to locomotion in one particular direction. These are the spatangoids, in some of which the spines all point backwards, imparting to the creatures somewhat the appearance of a hedgehog. The anus, furthermore, is commonly shifted back-

ward from the dorsal position until it comes to lie on the posterior border of the periphery, while conversely, the mouth may be located considerably forward of the mid-ventral position. Now it is of considerable interest to note that the region which is anterior is an ambulacrum, corresponding to a ray of the starfish, and that the *madreporite always lies in the inter-ambulacral area next to the right of the anterior ambulacrum*. If, therefore, we designate the different areas with relation to the madreporite as we have done in the starfish, it will be observed that *the anterior ambulacrum is c (III)¹¹ and this is the ray which was found to function as the 'physiological anterior' in Asterias*. These radii in the two cases are accordingly clearly analogous physiologically, but whether they are morphologically homologous, it would be hazardous to state, since the complications of embryological development make this point practically impossible to determine. The physiological relationship may be readily understood by comparing figure 9, which is a diagrammatic representation of a spatangoid, with figure 5 (p. 11) which similarly represents the starfish.

In holothurians the morphological and axial relations are so different from those of the forms we have been discussing that there would be little of value in a comparison. It is of interest to note, however, that Pearse ('08, p. 269) found in *Thyone* a preferential use of certain tentacles. As to locomotion however, he states (p. 264) that 'the animal may move in any direction,' and (p. 266) that

Individuals move with the posterior end in advance as often as with the anterior end, and although the long axis of the body is as a rule approximately parallel with the direction of locomotion, animals often move a long distance (as much as 12 cm.) with the body at right angles to the direction of movement, that is, they move straight toward the right or left.

¹¹ The anus consequently lies in the inter-ambulacral area (5) corresponding to the inter-radius *bc* of the starfish. In certain of the radial sea-urchins (e. g. *Strongylocentrotus*) the anus occupies an eccentric position in the periproct, lying nearer to the border opposite interambulacrum 5 (*bc*). A line drawn through ambulacrum III (*e*) and interambulacrum 5 (*bc*) therefore marks the beginning of a bilateral symmetry and presages the condition found in the spatangoids.

He apparently made no test, however, of the proportion of locomotion forward and backward in the absence of directive stimuli.

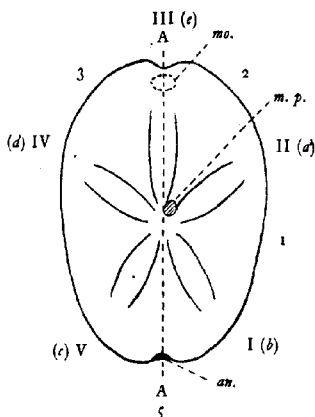


Fig. 9 Diagram of a spatangoid. 1—v, Lovén's designation of the ambulacral areas; (a)—(e), corresponding arms of the starfish with respect to the position of the madreporic plate, as designated by Jennings (cf. fig. 1); 1—5, interambulacral areas, according to Lovén. *an.*, anus; *mo.*, mouth; *m.p.*, madreporic plate; A.A., line marking bilateral symmetry of form. This diagram should be compared with fig. 5.

VI. CONCLUDING DISCUSSION

Granting that the observations which have been presented establish satisfactorily the fact of a 'physiological anterior' in the starfish—that a particular part tends to precede most often in locomotion—three possible explanations to account for this phenomenon suggest themselves. First, there may be a definite morphological relation between the bilateral larva and the adult which establishes what shall be 'anterior' in the latter; second, it may depend upon a proportional relationship in the length of the rays; and third, it may be related to the condition of some other set of organs, such as the nervous system or the water vascular system.

In considering the first of these theories we are at once confronted by the great complications which take place in the metamorphosis of the starfish, and which render extremely difficult the correlation of planes and the orientation of parts in the larva and in the adult. It is true that a number of years ago Goto ('98, p. 241) believed he had proven "a direct connection between the two principal planes in question, sagittal of brachiolaria and of adult," and "that this connection is that of exact coincidence." He believed his studies made it clear (p. 242) that

The sagittal plane of the larva cuts the disc of the star at right angles and passes through the water-pore and the centre of the disc, that is to say, the sagittal plane of the larva and the plane of bilateral symmetry of the star are coincident. The arms of the starfish may therefore be justly spoken of as the median ventral, the right and left dorsal, and the right and left ventral, arms. It need hardly be added that the oral side is anterior, and the aboral side posterior

as they are in the holothurian.

It is clearly evident that if this simple relationship were true it would serve nicely to explain the results on locomotion, since the plane of bilaterality with respect to direction of crawling practically coincides with that which he believes separates the symmetrical halves of the brachiolarian larva and the adult, at least if we consider the physiological 'anterior' to be in the general direction of arms *c* and *a* rather than exactly through *a*. Later researches have, however, apparently failed to corroborate Goto's conclusions, the complications of metamorphosis being much more difficult to unravel than would appear from his description, so that we are probably not justified in accepting this as an explanation for the preponderance of locomotion in one direction.

Similarly, the measurements which have been presented, although they appear to show a very slight correlation between mean length of arm and direction of locomotion, would seem to indicate that this explanation too must be rejected. Furthermore, if such a correlation actually exists, it will not serve as a satisfactory explanation, for we should still have to account for the proportional relationship of the arms.¹²

¹² Goto's contention, if true, would of course satisfactorily account for this.

The third suggestion is that the observed facts regarding locomotion may be explained by some peculiarity of the nervous or water vascular systems. As to the former of these, the nervous system appears to offer no peculiarity which could account for a differential in the locomotion. On the other hand, in the water vascular system we do find a special structure which breaks up the radial symmetry and which bears a positional relationship to the physiological differentiation observed in the locomotion. This structure is the madreporite, which connects the water vascular system with the exterior.

It will be recalled that whereas the experimental results established for *Asterias forbesi* a plane of physiological bilaterality through arm *e* and between *b* and *c* (cf. fig. 5), arm *a* was found to be most frequently the longest or one of the two longest (fig. 6 and table, p. 13), and also had the greatest mean length. These facts were shown in figure 7, which shows the axis-passing through arm *a* and between arms *c* and *d*. Preyer's experiments on other starfishes, on the other hand, indicated a plane passing between *e* and *a* (thus intersecting the madreporite) and through arm *c* (p. 26). The significant fact appears to be that all these data seem to indicate a plane passing through or near the madreporic plate. Proximity to the madreporite seems then to be associated in some way with a greater activity or other functional superiority of the tube feet on the arms so situated, for such is probably the reason for the preponderance of locomotion in this direction. Just why this should be so is not so clear, though it may be purely mechanical; this portion of the ambulacral system may be able to adjust itself more readily to changes in volume of contained water, consequent upon the activity of the ampullae and tube feet, because of its nearness to the outside reserve, that is, the outside water surrounding the starfish, just as a better supply is had near the mains of any water system than in the more remote branches.¹³

¹³ It would be interesting to determine whether there is possibly a difference in caliber of the radial tubes in different arms, and whether this is related to their nearness in origin to the stone canal.

VII. SUMMARY

The principal points brought out in this paper may be summarized as follows:

1. Experiments indicate that the starfish (*Asterias forbesi*, and probably other species), in the absence of directive stimuli, does crawl more frequently with a particular part of the body in advance, namely the part in proximity to the madreporite. This demonstrates a 'physiological anterior,' and a plane passed through the madreporite or one of the adjacent rays divides the animal into symmetrical physiological halves.

2. The correlation between direction of locomotion and mean arm length is doubtful and the results obtained are probably not significant.

3. A definite 'impulse' is established on account of which the starfish tends to crawl in the same general direction in successive trials.

4. There is a tendency for this impulse to shift or 'rotate' gradually around the body in one direction or the other.

5. After righting itself, the starfish more often crawls in the general direction of the rays opposite to those which it has used primarily in righting.

6. Preyer's results from his experiments fall substantially in line with those on *Asterias forbesi*.

7. The 'physiological anterior' of the starfish corresponds to anterior in the spatangoids, with respect to the position of the madreporite.

8. The position of the madreporite may perhaps be what determines 'anterior,' and it is possible that this may be from purely mechanical causes.

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ADDITIONAL DATA FOR THE STUDY OF SEX- LINKED INHERITANCE IN DROSOPHILA

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In a paper dealing with Data for the Study of Sex-linked Inheritance in *Drosophila* (Jour. Exp. Zool., vol. 13, no. 1, 1912) we described seven crosses in which three pairs of the sex-linked factors were involved. Three crosses that belonged to the same series were withdrawn because, as stated, the results were anomalous in certain points. It seemed almost certain that an error had crept in somewhere. The new results show, in fact, that these crosses are consistent with the other results concerning eye color, body color and wing characters. The new data, added to those of our former paper, to those of Morgan's paper for 1911, and to those of Dexter's paper that has just appeared, give numbers large enough to show the 'coupling strength' of some of the factors involved.

THE HEREDITY OF THREE CONTRASTED SEX-LINKED CHARACTERS

In the former paper (page 89) the second, third, and fourth combinations were the ones omitted. They are given here in sequence. The same symbols are used and the same method employed in writing out the analyses that were used before.

Short, red, black by long, white, yellow

This is the reciprocal of the cross already published (1912, page 90). When the female, LWY is mated to the male SRB all the female offspring are long, red, gray and all the males long, white, yellow. The numerical results for this and the F_2 generation are as follows:

	SRB ♂ by LWY ♀	
F ₁	LRN ♀ =	283
	LWY ♂ =	157
	LRN ♀ =	391
	LRN ♂ =	94
F ₂	LRB ♀ =	122
	LRB ♂ =	41
	SRN ♂ =	175
	SRB ♂ =	37
	LWY ♀ =	248
	LWY ♂ =	215
	LWBr ♀ =	86
	LWBr ♂ =	45
	SWY ♂ =	58
	SWBr ♂ =	10
	LRV ♀ =	5
	SRV ♂ =	1
	SRBr ♂ =	8
	LWN ♀ =	2
	LWN ♂ =	4

The analyses follow:

$$\begin{aligned} \text{LWbYBrX} &- \text{LWbYBrX} \\ \text{SRByBrX} &- \text{SWbyBr} \end{aligned}$$

F ₁	LWbYBrX — SRByBrX	Long, red, normal.
	LWbYBrX — SWbyBr	Long, white, yellow.

Gametes of F ₁	LWbyBrX — LWbYBrX — SRByBrX — SRBYBrX	} Eggs
	SWbyBrX — SWbYBrX — LRByBrX — LRBYBrX	
	LWbyBrX — LWbYBrX — SWbyBr — SWbyBr	Sperm

	Females		Males	
F ₂	LWBr	2	LWBr	1
	LWY	6	LWY	3
	LRB	2	LRB	1
	LRN	6	LRN	3

There were 20 cases of crossing-over counting both males and females in a total of 662, or 1 to 33. It is legitimate to count both sexes here because both males and females lack the

dominant characters (C and R). The number of cross-overs is relatively high. The converse cross gave 1 to 97 (see Jour. Exp. Zool., p. 91, vol. 13, 1912).

Of the cross-overs there were 14 in one direction to 6 in the opposite. Moreover, it is the short, red, browns and yellows, often assumed to be the less viable combination, that give the larger number.

Short, red, yellow by long, white, black

The two crosses recorded under this heading, both absent from the preceding paper, were Nos. 3, and 4 of the series (page 89).

When the female LWB is crossed to the male SRY all the female offspring are long, red, gray and all the males long, white, gray. The numerical results follow:

	SRY ♂ by LWB ♀
F ₁	LRN ♀ = 80
	LWN ♂ = 78
	LRN ♀ = 572
	LRB ♀ = 191
	LWN ♀ = 586
	LWN ♂ = 314
F ₂	LWB ♀ = 151
	LWB ♂ = 99
	SWN ♂ = 112
	SWB ♂ = 33
	LRY ♂ = 154
	LRBr ♂ = 66
	SRY ♂ = 295
	SRBr ♂ = 165
	SRN ♂ = 11
	LWY ♂ = 1

The analysis follows:

	LWByBrX — LWByBrX
	SRbYBrX — SWbYBr
F ₁	LWByBrX — SRbYBrX
	LWByBrX — SWbYBr

Gametes of F_1	LWBYBrX — LWByBrX — SRbYBrX — SRbyBrX	} Eggs
	SWBYBrX — SWBYBrX — LRbYBr — LBbyBrX	
	LWBYBrX — LWByBrX — SWbYBr — SWbyBr	
		Sperm
<hr/>		
	Females	Males
F_2	LWN 6	LWN 3 SWN 3
	LRN 6	SRYel 3 LRY 3
	LWB 2	LWB 1 SWB 1
	LRB 2	SRBr 1 LWB 1

There were 13 cases of crossing over in the 1178 males or 1 to 90. Of these crossings 11 were in one direction, and 1 in the other.

The reciprocal cross gave the following results:

SRY ♀ by LWB ♂		
F_1	LRN ♀	= 9
	SRY ♂	= 18 ¹
F_2	LRN ♀	= 145
	LRB ♀	= 52
	LWN ♂	= 150
	LWB ♂	= 44
	SRN ♀	= 45
	SRB ♀	= 21
	SWN ♂	= 58
	SWB ♂	= 16
	LRY ♀	= 72
	LRY ♂	= 71
	LRBr ♀	= 21
	LRBr ♂	= 13
	SRY ♂	= 107
	SRY ♀	= 88
	SRBr ♂	= 41
	SRBr ♀	= 64
	SRN ♂	= 11
	SRB ♂	= 5
	LWY ♂	= 3
	LRN ♂	= 1

¹ One LWY found in F_1

There were 20 cases of cross-overs amongst 500 males, or 1 in 25.

The analysis follows:

F ₁	SRbYBrX — SRbYBrX				Eggs		
	LWbYBrX — SWbYBr						
	SRbYBrX		LWbYBrX				
	SRbYBrX		SWbYBr				
Gametes of F ₁	SRbYBrX — SRbYBrX		LWbYBrX — LWbYBrX		Sperm		
	LRbYBrX — LRbYBrX		SWbYBrX — SWbYBrX				
	SRbYBrX		SWbYBr				
	SRbYBrX		SWbYBr				
Females			Males				
SRBr	1	LRBr	1	SRBr	1	LRBr	1
SRY	3	LYR	3	SRYel	3	LYRel	3
SRBl	1	LRBl	1	LWBl	1	SWBl	1
SRN	3	LRN	3	LWN	3	SWN	3

This last cross gave, the first time it was tried, the results recorded below. These results were not published because of the very high number of the cross-over class, SRN = 17. In the repetition of the experiment the class is again large, but not so excessive as in the former instance. As there were no grounds for suspecting these results in other respects we feel that the only fair course is to add them here. In the sum totals, however, they have been omitted.

	SRY	φ	by	LWB	σ^2
F_1	LRN	φ	=	76	
	SRY	σ^2	=	86	
	LRN	φ	=	93	
	LRB	φ	=	41	
	LWN	σ^2	=	101	
	LWB	σ^2	=	33	
	SRN	φ	=	25	
	SRB	φ	=	9	
	SWN	σ^2	=	30	
	SWB	σ^2	=	13	
F_2	LRV	φ	=	48	
	LRV	σ^2	=	44	
	LRBr	φ	=	9	
	LRBr	σ^2	=	3	
	SRY	φ	=	47	
	SRY	σ^2	=	51	
	SRBr	φ	=	17	
	SRBr	σ^2	=	23	
	SRN	σ^2	=	17	
	SRB	σ^2	=	1	

The linkage of RY and WB

In order to obtain a larger amount of data for linkage of R and Y versus W and B, when the result is not complicated by other factors, we repeated one of our former crosses that was too small for the matter in hand. Long, red, yellow females were crossed with long, white, black males and gave LRN ♀ and LRY ♂. The numerical results for F₁ and F₂ are given below.

	LRN ♀ LWB ♂
F ₁	LRN ♀ = 622
	LRY ♂ = 521
F ₂	LRN and Bl ♀ 2034
	LRY and Br ♀ 1740
	LRY and Br ♂ 1682
	LWN and Bl ♂ 1561
	LRN and Bl ♂ 43
	LWY and Br ♂ 10

In the total of 3243 males there were 53 cases of cross-over or 1:61. In the former experiment there were 260 males and no cross-overs.

In order to obtain still more data some of the F₁ LRN ♀ of the last cross were mated with the double recessive LWY ♂. Since the latter contains both of the recessive factors, w and b, all the offspring of the cross, both males and females, may be counted. The results were as follows:

LWN ♂ and ♀	= 2731
LRY ♂ and ♀	= 2923
LRN ♂ and ♀	= 43
LWY ♂ and ♀	= 34

In a total of 5644 there were 77 cross-overs or 1 to 73.3.

GENERAL CONCLUSIONS

It is not our intention to discuss here the many questions that arise in connection with these results, but rather to put the data on record for future reference. There are three topics, however, that require summing up:

1. The total record of crossing-over for the combination YW and BR and the reciprocal.
2. The total record of crossing-over for LW and SR and the reciprocal.
3. The record of crossing-over for LY and SR and the reciprocal.

In all three are six records of crossing over of YW and BR. The simplest cases are those in which both parents have long wings. We have Dexter's large count, counts in two preceding papers (Morgan '11, Morgan and Cattell '12) and the counts of the present paper.

	F ₂	CROSS-OVERS	RATIO
Dexter.....	16002	189	1 : 84.7
Morgan.....	3253	5	1 : 650.0
Morgan-Cattell.....	1806	15	1 : 120.4
Morgan-Cattell.....	8807	130	1 : 68.4
Total.....	29958	339	1 : 88.3

In these counts, the second in the table gave anomalous results, the cross-overs falling below the other cases. The last three cases contain brown which has a high mortality; yet the total result is not far from Dexter's results. In the next table the results are complicated by the presence of short wings, which run behind long wings in some combinations, particularly where short, yellow, white is expected.

	F ₂	CROSS-OVERS	RATIO
W. H. Morgan, 1912.....	4890	55	1 : 89
Morgan-Cattell, 1912.....	10748	80	1 : 134
Total.....	15538	135	1 : 115

In this case the number of cross-overs is considerably smaller than in the last, but since many classes are included and some with high mortality the former estimate is probably more correct.

If we add the two results together we get a total of 35496 counts in addition to the 474 cross-overs, which, on the basis of calculations here employed, gives a ratio of 1 to 95. Whether the difference in these ratios obtained at different times stand for variations in the process itself, or whether they represent chance results in the sense that for such a relatively rare event the probable error will account for the differences can be more profitably discussed at another time when the question of the gametic ratios has been more fully studied.

The gametic ratio of long and miniature wings versus red and white eyes (LW and SR) gives more even results, because the crossing-over is much more frequent and smaller numbers give significant results. In our former paper ('12) there were 6829 cases of no crossing-over to 3573 cross-overs; a ratio of 1:1.8. In the present paper there were 1835 cases of no crossing to 816 cross-overs; a ratio of 1:2.2. In the paper of 1911 there were 3177 cases of no crossing to 1713 cross-overs; a ratio of 1:1.8.

NO CROSSING	CROSS-OVERS	RATIO
6829	3573	1 : 1.9
1835	816	1 : 2.2
3177	1713	1 : 1.8
11841	6102	1 : 1.94

Adding these cases together, the linkage ratio is 1:1.94. This means that the chance is about twice as great that the grand-parental combination will hold as that it will break. These results will need to be corrected for viability, and for other disturbances as well, but there can be little doubt that the results give approximately the gametic ratio for this combination. No attempt has been made here to separate those cases where LW

and SR formed one pair and SW and LR another pair, because the results, as far as they go at present, do not seem to give very significant difference in relation to which way the original couple was made, but here again a more critical examination may be called for. In these latter figures we have omitted the 'small classes' containing the cross-overs of YW and RG. Their omission does not affect materially the sum total although they should be included, with certain corrections that can not now be made.

Finally, these same data give the linkage ratio of S and L to B' (black and normal) and b (yellow and brown). This is the linkage between the factor in question for wings and that for body color. We should expect that this ratio would closely approximate to the last since the first calculation showed that crossing-over between eye color and body color occurred only once in 88.3 cases.

In the paper of 1911, there were 1713 cross-overs to 3175 coupled cases, a ratio of 1:2.4. In the 1912 paper (Morgan-Cattell) there were 2921 cross-overs to 5383 coupled cases, a ratio of 1:1.8. In the present paper there are 527 cross-overs to 1155 coupled cases, a ratio of 1:2.2. The sum of all these cross-overs is 5161 and of the coupled cases, 9713, a ratio of about 1:1.98.

	CROSS-OVERS	COUPLES	RATIO
Morgan, 1911.....	1713	3175	1 : 2.4
Morgan-Cattell, 1912.....	2921	5383	1 : 1.8
Morgan-Cattell, 1912.....	527	1155	1 : 2.2
Total.....	5161	9713	1 : 1.88

In these counts the 'small classes' are not included. The ratio of the total count is 1:1.88, which is almost identical with the ratio 1:1.94 for wings and eye color.

The difference to be expected between the gametic ratios of the last two cases (wings and eye color and wings and body color) would, on the basis of the first case considered (eye color and

body-color) be only 1 to 88. This difference is so small that it affects the data given above only slightly. It could be accurately estimated only by adding in to the two last calculations the cross-over and coupled cases given in the 'small classes,' but this addition involves the consideration of another matter, namely, double-crossing-over. Without going further into the details of this question the addition of the small classes would be misleading. As the matter is especially considered by Mr. A. H. Sturtevant in a paper appearing at the same time as this one, the whole discussion may be left to his handling.

THE LINEAR ARRANGEMENT OF SIX SEX-LINKED
FACTORS IN DROSOPHILA, AS SHOWN BY
THEIR MODE OF ASSOCIATION

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HISTORICAL

The parallel between the behavior of the chromosomes in reduction and that of Mendelian factors in segregation was first pointed out by Sutton ('02) though earlier in the same year Boveri ('02) had referred to a possible connection (loc. cit., footnote 1, p. 81). In this paper and others Boveri brought forward considerable evidence from the field of experimental embryology indicating that the chromosomes play an important rôle in development and inheritance. The first attempt at connecting any given somatic character with a definite chromosome came with McClung's ('02) suggestion that the accessory chromosome is a sex-determiner. Stevens ('05) and Wilson ('05) verified this by showing that in numerous forms there is a sex chromosome, present in all the eggs and in the female-producing sperm, but absent, or represented by a smaller homologue, in the male-producing sperm. A further step was made when Morgan ('10) showed that the factor for color in the eyes of the fly *Drosophila ampelophila* follows the distribution of the sex-chromosome already found in the same species by Stevens ('08). Later, on the appearance of a sex-linked wing mutation in *Drosophila*, Morgan ('10 a, '11) was able to make clear a new point. By crossing white eyed, long winged flies to those with red eyes and rudimentary wings (the new sex-linked character) he obtained, in F_2 , white eyed rudimentary winged flies. This could happen

only if 'crossing over' is possible; which means, on the assumption that both of these factors are in the sex-chromosomes, that an interchange of materials between homologous chromosomes occurs (in the female only, since the male has only one sex-chromosome). A point not noticed at this time came out later in connection with other sex-linked factors in *Drosophila* (Morgan '11 d). It became evident that some of the sex-linked factors are associated, i.e., that crossing over does not occur freely between some factors, as shown by the fact that the combinations present in the F_1 flies are much more frequent in F_2 than are new combinations of the same characters. This means, on the chromosome view, that the chromosomes, or at least certain segments of them, are more likely to remain intact during reduction than they are to interchange materials.¹ On the basis of these facts Morgan ('11 c, '11 d) has made a suggestion as to the physical basis of coupling. He uses Janssens' ('09) chiasmatype hypothesis as a mechanism. As he expresses it (Morgan '11 c):

If the materials that represent these factors are contained in the chromosomes, and if those that "couple" be near together in a linear series, then when the parental pairs (in the heterozygote) conjugate like regions will stand opposed. There is good evidence to support the view that during the strepsinema stage homologous chromosomes twist around each other, but when the chromosomes separate (split) the split is in a single plane, as maintained by Janssens. In consequence, the original materials will, for short distances, be more likely to fall on the same side of the split, while remoter regions will be as likely to fall on the same side as the last, as on the opposite side. In consequence, we find coupling in certain characters, and little or no evidence at all of coupling in other characters, the difference depending on the linear distance apart of the chromosomal materials that represent the factors. Such an explanation will account for all the many phenomena that I have observed and will explain equally, I think, the other cases so far described. The results are a simple mechanical result, of the location of the materials in the chromosomes, and of the method of union of homologous chromosomes, and the proportions that result are not so much the expression of a numerical system as of the relative location of the factors in the chromosomes.

¹ It is interesting to read, in this connection, Lock's ('06, p. 248-253) discussion of the matter.

SCOPE OF THIS INVESTIGATION

It would seem, if this hypothesis be correct, that the proportion of 'cross-overs' could be used as an index of the distance between any two factors. Then by determining the distances (in the above sense) between A and B and between B and C, one should be able to predict AC. For, if proportion of cross-overs really represents distance, AC must be approximately, either AB plus BC, or AB minus BC, and not any intermediate value. From purely mathematical considerations, however, the sum and the difference of the proportion of cross-overs between A and B and those between B and C are only *limiting* values for the proportion of cross-overs between A and C. By using several pairs of factors one should be able to apply this test in several cases. Furthermore, experiments involving three or more sex-linked allelomorphic pairs together should furnish another and perhaps more crucial test of the view. The present paper is a preliminary report of the investigation of these matters.

I wish to thank Dr. Morgan for his kindness in furnishing me with material for this investigation, and for his encouragement and the suggestions he has offered during the progress of the work. I have also been greatly helped by numerous discussions of the theoretical side of the matter with Messrs. H. J. Muller, E. Altenburg, C. B. Bridges, and others. Mr. Muller's suggestions have been especially helpful during the actual preparation of the paper.

THE SIX FACTORS CONCERNED

In this paper I shall treat of six sex-linked factors and their inter-relationships. These factors I shall discuss in the order in which they seem to be arranged.

B stands for the black factor. Flies recessive with respect to it (b) have yellow body color. The factor was first described and its inheritance given by Morgan ('11 a).

C is a factor which allows color to appear in the eyes. The white eyed fly (first described by Morgan '10) is now known to be always recessive with respect both to C and to the next factor.

O. Flies recessive with respect to O(o) have eosin eyes. The relation between C and O has been explained by Morgan in a paper now in print and about to appear in the Proceedings of the Academy of Natural Sciences in Philadelphia.

P. Flies with p have vermilion eyes instead of the ordinary red (Morgan '11 d).

R. This and the next factor both affect the wings. The normal wing is RM. The rM wing is known as miniature, the Rm as rudimentary, and the rm as rudimentary-miniature. This factor R is the one designated L by Morgan ('11 d) and Morgan and Cattell ('12). The L of Morgan's earlier paper ('11) was the next factor.

M. This has been discussed above, under R. The miniature and rudimentary wings are described by Morgan ('11 a).

The relative position of these factors is B, $\frac{C}{O}$, P, R, M. C and O are placed at the same point because they are completely linked. Thousands of flies had been raised from the cross CO (red) by co (white) before it was known that there were two factors concerned. The discovery was finally made because of a mutation and not through any crossing over. It is obvious, then, that unless coupling strength be variable, the same gametic ratio must be obtained whether, in connection with other allelomorphic pairs, one uses CO (red) as against co (white), Co (eosin) against co (white), or CO (red) against Co (eosin) (the cO combination is not known).

METHOD OF CALCULATING STRENGTH OF ASSOCIATION

In order to illustrate the method used for calculating the gametic ratio I shall use the factors P and M. The cross used in this case was, long winged, vermilion-eyed female by rudimentary winged, red-eyed male. The analysis and results are seen in table 1.

It is of course obvious from the figures that there is something peculiar about the rudimentary winged flies, since they appear in far too small numbers. This point need not detain us here, as it always comes up in connection with rudimentary crosses,

TABLE I

	Long vermilion	♀—MpX MpX
	Rudimentary red	♂—mPX
F ₁	MpX mPX—long red	♀
	MpX —long vermilion	♂
Gametes F ₁	Eggs —MPX mPX MpX mpX	
	Sperm—MpX	
F ₂	MPX MpX	} —long red ♀—451
	mPX MpX	
	MpX MpX	} —long vermilion ♀—417
	mpX MpX	
	MPX	—long red ♂—105
	mPX	—rudimentary red ♂—33
	MpX	—long vermilion ♂—316
	mpX	—rudimentary vermilion ♂—4

and is being investigated by Morgan. The point of interest at present is the linkage. In the F₂ generation the original combinations, red rudimentary and vermilion long, are much more frequent in the males (allowing for the low viability of rudimentary) than are the two new or cross-over combinations, red long and vermilion rudimentary. It is obvious from the analysis that no evidence of association can be found in the females, since the M present in all female-producing sperm masks m when it occurs. But the ratio of cross-overs in the gametes is given without complication by the F₂ males, since the male-producing sperm of the F₁ male bore no sex-linked genes. There are in this case 349 males in the non-cross-over classes and 109 in the cross-overs. The method which has seemed most satisfactory for expressing the relative position of factors, on the theory proposed in the beginning of this paper, is as follows. The unit of 'distance' is taken as a portion of the chromosome of such length that, on the average, one cross-over will occur in it out of every 100 gametes formed. That is, percent of cross-overs is used as an index of distance. In the case of P and M there occurred 109 cross-overs in 405 gametes, a ratio of 26.9 in 100; 26.9, the per cent of cross-overs, is considered as the 'distance' between P and M.

TABLE 2

FACTORS CONCERNED	PROPORTION OF CROSS-OVERS	PER CENT OF CROSS-OVERS
BCO.....	193 16287	1.2
BO.....	2 373	0.5
BP.....	1464 4551	32.2
BR.....	115 324	35.5
BM.....	260 693	37.6
COP.....	224 748	30.0
COR.....	1643 4749	34.6
COM.....	76 161	47.2
OP.....	247 836	29.4
OR.....	183 538	34.0
OM.....	218 404	54.0
CR.....	236 829	28.5
CM.....	112 333	33.6
B(C, O).....	214 21736	1.0
(C, O)P.....	471 1584	29.7
(C, O)R.....	2002 6116	33.7
(C, O)M.....	496 898	45.2
PR.....	17 573	3.0
PM.....	109 405	26.9

THE LINEAR ARRANGEMENT OF THE FACTORS

Table 2 shows the proportion of cross-overs in those cases which have been worked out. The detailed results of the crosses involved are given at the end of this paper. The 16287 cases for B and CO are from Dexter ('12). Inasmuch as C and O are completely linked I have added the numbers for C, for O, and for C and O taken together, giving the total results in the lines beginning (C, O) P, B (C, O), etc., and have used these figures, instead of the individual C, O, or CO results, in my calculations. The fractions in the column marked 'proportion of cross-overs' represent the number of cross-overs (numerator) to total available gametes (denominator).

As will be explained later, one is more likely to obtain accurate figures for distances if those distances are short, i.e., if the asso-

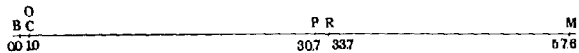


Diagram 1

ciation is strong. For this reason I shall, in so far as possible, use the percent of cross-overs between adjacent points in mapping out the distances between the various factors. Thus, B (C, O), (C, O) P, PR, and PM form the basis of diagram 1. The figures on the diagram represent calculated distances from B.

Of course there is no knowing whether or not these distances as drawn represent the actual relative spacial distances apart of the factors. Thus the distance CP may in reality be shorter than the distance BC, but what we do know is that a break is far more likely to come between C and P than between B and C. Hence, either CP is a long space, or else it is for some reason a weak one. The point I wish to make here is that we have no means of knowing that the chromosomes are of uniform strength, and if there are strong or weak places, then that will prevent our diagram from representing actual relative distances—but, I think, will not detract from its value as a diagram.

Just how far our theory stands the test is shown by table 3, giving observed per cent of cross-overs, and distances as calcu-

lated from the figures given in the diagram of the chromosome. Table 3 includes all pairs of factors given in table 2 but not used in the preparation of the diagram.

It will be noticed at once that the long distances, BM, and (C, O)M, give smaller percent of cross-overs than the calculation calls for. This is a point which was to be expected, and will be discussed later. For the present we may dismiss it with the statement that it is probably due to the occurrence of two breaks in the same chromosome, or 'double crossing over.' But in the case of the shorter distances the correspondence with expectation is perhaps as close as was to be expected with the small numbers that are available. Thus, BP is 3.2 less than BR, the difference

TABLE 3

FACTORS	CALCULATED DISTANCE	OBSERVED PER CENT OF CROSS-OVERS
BP.....	30.7	32.2
BR.....	33.7	35.5
BM.....	57.6	37.6
(C, O)R.....	32.7	33.7
(C, O)M.....	56.6	45.2

expected being 3.0. (C, O)R is less than BR by 1.8 instead of by 1.0. It has actually been found possible to predict the strength of association between two factors by this method, fair approximations having been given for BR and for certain combinations involving factors not treated in this paper, before the crosses were made.

DOUBLE CROSSING OVER

On the chiasmatype hypothesis it will sometimes happen, as shown by Dexter ('12) and intimated by Morgan ('11 d) that a section of, say, maternal chromosome will come to have paternal elements at both ends, and perhaps more maternal segments beyond these. Now if this can happen it introduces a complication into the results. Thus, if a break occurs between B and P, and another between P and M, then, unless we can follow P also, there will be no evidence of crossing over between B and

M, and the fly hatched from the resulting gamete will be placed in the non-cross-over class, though in reality he represents two cross-overs. In order to see if double crossing over really does occur it is necessary to use three or more sex-linked allelomorphic pairs in the same experiment. Such cases have been reported by Morgan ('11 d) and Morgan and Cattell ('12) for the factors B, CO, and R. They made such crosses as long gray red by miniature yellow white, and long yellow red by miniature gray white, etc. The details and analyses are given in the original papers, and for our present purpose it is only the flies that are available for observations on double crossing over that are of interest. Table 4 gives a graphical representation of what happened in the 10495 cases.

Double crossing over does then occur, but it is to be noted that the occurrence of the break between B and CO tends to prevent that between CO and R (or vice versa). Thus where B and CO did not separate, the gametic ratio for CO and R was about 1 to 2, but in the cases where B and CO did separate it was about 1 to 6.5.

Three similar cases from my own results, though done on a smaller scale, are given in the table at the end of this paper. The results are represented in tables 5, 6 and 7.

TABLE 4

NO CROSSING OVER	SINGLE CROSSING OVER	DOUBLE CROSSING OVER
$\begin{array}{ c } \hline B \\ \hline CO \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline CO \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline CO \\ \hline R \\ \hline \end{array}$
6972	3454	60

TABLE 5

NO CROSSING	SINGLE CROSSING OVER	DOUBLE CROSSING OVER
$\begin{array}{ c } \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$
194	102	11

TABLE 6

NO CROSSING	SINGLE CROSSING OVER		DOUBLE CROSSING OVER
$\begin{array}{ c } \hline B \\ \hline O \\ \hline M \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline M \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline M \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline M \\ \hline \end{array}$
278	160	1	0

TABLE 7

$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$	$\begin{array}{ c } \hline B \\ \hline O \\ \hline P \\ \hline R \\ \hline \end{array}$
393	203	19	6	2	1	1	0

It will be noted that here also the evidence, so far as it goes, indicated that the occurrence of one cross-over makes another one less likely to occur in the same gamete. In the case of BOPR there was an opportunity for triple crossing over, but it did not occur. Of course, on the view here presented there is no reason why it should not occur, if enough flies were raised. An examination of the figures will show that it was not to be expected in such small numbers as are here given. So far as I know there is, at present, no evidence that triple crossing over takes place, but it seems highly probable that it will be shown to occur.²

Unfortunately, in none of the four cases given above are two comparatively long distances involved, and in only one are there enough figures to form a fair basis for calculation, so that it seems as yet hardly possible to determine how much effect double crossing over has in pulling down the observed percent of cross-overs in the case of BM and (C, O)M. Whether or not this effect is partly counter-balanced by triple crossing over must also remain unsettled as yet. Work now under way should furnish answers to both these questions.

² A case of triple crossing over within the distance CR was observed after this paper went to press.

TABLE 8

(The meaning of the phrase 'proportion of cross-overs' is given on p. 45)

- BO. P₁: gray eosin ♀ × yellow red ♂
 F₁: gray red ♀ × gray eosin ♂
 F₂: ♀ ♀, g.r. 241, g.e. 196
 ♂ ♂, g.r. 0, g.e. 176, y.r. 195, y.e. 2

Proportion of cross-overs, $\frac{2}{373}$

- BP. P₁: gray red ♀ × yellow vermillion ♂
 F₁: gray red ♀ × gray red ♂
 F₂: ♀ ♀, g.r. 98;
 ♂ ♂, g.r. 59, g.v. 16, y.r. 24, y.v. 33
 Back cross, F₁ gray red ♀ × yellow vermillion ♂ ♂
 F₂: ♀ ♀, g.r. 31, g.v. 11, y.r. 12, y.v. 41
 ♂ ♂, g.r. 23, g.v. 13, y.r. 8, y.v. 21
 P₁: gray vermillion ♀ × yellow red ♂
 F₁: gray red ♀ × gray vermillion ♂
 F₂: ♀ ♀, g.r. 199, g.v. 182
 ♂ ♂, g.r. 54, g.v. 149, y.r. 119, y.v. 41
 P₁: yellow vermillion ♀ × gray red ♂
 F₁: gray red ♀ × yellow vermillion ♂
 F₂: ♀ ♀, g.r. 472, g.v. 240, y.r. 213, y.v. 414
 ♂ ♂, g.r. 385, g.v. 186, y.r. 189, y.v. 324
 F₁: gray vermillion × yellow red (sexes not recorded)
 F₁: gray red ♀ ♀. These were mated to yellow vermillion ♂ ♂ of other stock
 F₂: ♀ ♀, g.r. 50, g.v. 96, y.r. 68, y.v. 41
 ♂ ♂, g.r. 44, g.v. 105, y.r. 86, y.v. 47

Proportion of cross-overs, adding ♀ ♀ from BOPR (below), $\frac{1464}{4551}$

- BR. P₁ miniature yellow ♀ × long-gray ♂
 F₁: long gray ♀ × miniature yellow ♂
 F₂: ♀ ♀ l.g. 14, l.y. 2, m.g. 7, m.y. 6;
 ♂ ♂ l.g. 10, l.y. 1, m.g. 6, m.y. 8.
 P₁: long yellow ♀ × miniature gray ♂
 F₁: long gray ♀ × long yellow ♂
 F₂: ♀ ♀, l.g. 148, l.y. 130
 ♂ ♂, l.g. 51, l.y. 82, m.g. 89, m.y. 48

Proportion of cross-overs, $\frac{115}{324}$

TABLE 8 (continued)

BM.	P ₁ : long yellow ♀ × rudimentary gray ♂
	F ₁ : long gray ♀ × long yellow ♂
	F ₁ : ♀ ♀, l.g. 591, l.y. 549
	♂ ♂, l.g. 228, l.y. 371, r.g. 20, r.y. 3
	P ₁ : long gray ♀ × rudimentary yellow ♂
	F ₁ : long gray ♀ × long gray ♂
	F ₂ : ♀ ♀, l.g. 152
	♂ ♂, l.g. 42, l.y. 29, r.g. 0, r.y. 0
	Proportion of cross-overs, $\frac{260}{693}$
COP.	P ₁ : vermilion ♀ × white ♂
	F ₁ : red ♀ × vermilion ♂
	F ₂ : ♀ ♀, r. 320, v. 294
	♂ ♂, r. 86, v. 206, w. 211
	(7 of the vermilion ♀ ♀ known from tests to be CC, 2 known to be Cc. 7 white ♂ ♂ Pp, 2 pp.)
	Back cross, F ₁ red ♀ ♀ from above × white ♂ ♂, gave
	F ₂ : ♀ ♀, r.195, w. 227,
	♂ ♂, r. 66, v. 164, w. 184
	Out cross, F ₁ ♀ ♀ as above × white ♂ ♂ recessive in P, gave
	F ₂ : ♀ ♀, r. 35, v. 65, w. 98
	♂ ♂, r. 33, v. 75, w. 95
	Proportion of cross-overs, $\frac{224}{748}$
COR.	P ₁ : miniature white ♀ × long red ♂
	F ₁ : long red ♀ × miniature white ♂
	F ₂ : ♀ ♀, l.r. 193, l.w. 109, m.r. 124, m.w. 208
	♂ ♂, l.r. 202, l.w. 114, m.r. 123, m.w. 174
	P ₁ : long white ♀ × miniature red ♂
	F ₁ : long red ♀ × long white ♂
	F ₂ : ♀ ♀ l.r. 194, l. w. 160
	♂ ♂ l.r. 52, l. w. 124, m.r. 97, m.w. 41
	Proportion of cross-overs, $\frac{563}{1561}$; or, adding such available figures from
	Morgan ('11 d) and Morgan and Cattell ('12) as are not complicated
	by the presence of yellow or brown flies, $\frac{1643}{4749}$
COM.	P ₁ : long white ♀ × rudimentary red ♂
	F ₁ : long red ♀ × long white ♂
	F ₂ : ♀ ♀, l.r. 157, l.w. 127
	♂ ♂, l.r. 74, l.w. 82, ru.r. 3, ru.w. 2
	Proportion of cross-overs, $\frac{76}{161}$

TABLE 8 (continued)

- OP. P₁: black red ♀ × black eosin-vermilion ♂
 F₁: black red ♀ × black red ♂
 F₂: (all black), ♀ ♀, r. 885
 ♂ ♂, r. 321, v. 125, e. 122, e.-v. 268

Proportion of cross-overs, $\frac{247}{836}$

- OR. P₁: long red ♀ × miniature eosin ♂
 F₁: long red ♀ × long red ♂
 F₂: ♀ ♀, l.r. 408
 ♂ ♂, l.r. 145, l.e. 67, m.r. 70, m.e. 100
 P₁: long eosin ♀ × miniature red ♂
 F₁: long red ♀ × long eosin ♂
 F₂: ♀ ♀, l.r. 100, l.e. 95
 ♂ ♂, l.r. 27, l.e. 54, m.r. 56, m.e. 19

Proportion of cross-overs, $\frac{183}{538}$

- OM. P₁: long eosin ♀ × rudimentary red ♂
 F₁: long red ♀ × long eosin ♂
 F₂: ♀ ♀, l.r. 368, l.e. 266
 ♂ ♂, l.r. 194, l.e. 146, ru.r. 40, ru.e. 24

Proportion of cross-overs, $\frac{218}{404}$

- CR. P₁: long white ♀ × miniature eosin ♂
 F₁: long eosin ♀ × long white ♂
 F₂: ♀ ♀, l.e. 185, l.w. 205
 ♂ ♂, l.e. 54, l.w. 147, m.e. 149, m.w. 42
 P₁: long eosin ♀ × miniature white ♂
 F₁: long eosin ♀ × long eosin ♂
 F₂: ♀ ♀, l.e. 527
 ♂ ♂, l.e. 169, l.w. 85, m.e. 55, m.w. 128

Proportion of cross-overs, $\frac{236}{829}$

- CM. P₁: long white ♀ × rudimentary eosin ♂
 F₁: long eosin ♀ × long white ♂
 F₂: ♀ ♀, l.e. 328, l.w. 371
 ♂ ♂, l.e. 112, l.w. 217, ru.e. 4, ru.w. 0

Proportion of cross-overs, $\frac{112}{333}$

TABLE 8 (continued)

- PR. P₁: long vermillion (yellow) ♀ × miniature red (yellow) ♂
 F₁: long red yellow ♀ × long vermillion yellow ♂
 F₂: (all y.) ♀ ♀, l.r. 138, l.v. 110
 ♂ ♂, l.r. 8, l.v. 117, m.r. 97, m.v. 1
 P₁: long vermillion (gray) ♀ × miniature red ♂
 F₁: long red ♀ × long vermillion ♂
 F₂: ♀ ♀, l.r. 116, l.v. 110
 ♂ ♂, l.r. 2, l.v. 81, m.r. 96, m.v. 1
 P₁: miniature red ♀ × long vermillion ♂
 F₁: long red ♀ × miniature red ♂
 F₂: ♀ ♀, l.r. 45, m.r. 49
 ♂ ♂, l.r. 1, l.v. 27, m.r. 26, m.v. 0
 F₁: long red ♀ ♀ from above × miniature red ♂ ♂ of other stock, gave
 F₂: ♀ ♀, l.r. 74, m.r. 52
 ♂ ♂, l.r. 3, l.v. 66, m.r. 46, m.v. 1
 Proportion of cross-overs, $\frac{17}{573}$
- PM. P₁: long vermillion ♀ × rudimentary red ♂
 F₁: long red ♀ × long vermillion ♂
 F₂: ♀ ♀, l.r. 451, l.v. 417
 ♂ ♂, l.r. 105, l.v. 316, ru.r. 33, ru.v. 4
 Proportion of cross-overs, $\frac{109}{405}$
- OPR. P₁: long vermillion ♀ × miniature eosin ♂
 F₁: long red ♀ × long vermillion ♂
 F₂: ♀ ♀, l.r. 205, l.v. 182
 ♂ ♂, l.r. 1, l.v. 109, l.e. 8, l.e.-v. 53, m.r. 49, m.v. 3, m.e. 85, m.e.-v. 0
- BOM. P₁: long red yellow ♀ × rudimentary eosin gray ♂
 F₁: long red gray ♀ × long red yellow ♂
 F₂: ♀ ♀, l.r.g. 530, l.r.y. 453
 ♂ ♂, l.r.g. 1, l.r.y. 274, l.e.g. 156, l.e.y. 0, ru.r.g. 0, ru.r.y. 4, ru.e.g. 4,
 ru.e. y. 0
- BOPR. P₁: long vermillion brown ♀ × miniature eosin black ♂
 F₁: long red black ♀ × long vermillion brown ♂
 F₂: ♀ ♀, l.r.bl. 305, l.r.br. 113, l.v.bl. 162, l.v.br. 256
 ♂ ♂, l.r.bl. 0, l.r.br. 2, l.v.bl. 3, l.v.br. 185, l.e.bl. 9, l.e.br. 0, l.e.-v.bl.
 127, l.e.-v.br. 0, m.r.bl. 1, m.r.br. 76, m.v.bl. 1, m.v.br. 10, m.e.bl.
 208, m.e.br. 3, m.e.-v.bl. 0, m.e.-v.br. 0

POSSIBLE OBJECTIONS TO THESE RESULTS

It will be noted that there appears to be some variation in coupling strength. Thus, I found (CO)R to be 36.7; Morgan and Cattell obtained the result 33.9; for OR I got 34.0, and for CR, 28.5. The standard error for the difference between (CO)R (all figures) and CR is 1.84 per cent, which means that a difference of 5.5 per cent is probably significant (Yule '11, p. 264). The observed difference is 6.1 per cent, showing that there is some complication present. Similarly, BM gave 37.6, while OM gave 54.0—and BOM gave 36.7 for BM, and 36.5 for OM. There is obviously some complication in these cases, but I am inclined to think that the disturbing factor discussed below (viability) will explain this. However, experiments are now under way to test the effect of certain external conditions on coupling strength. It will be seen that on the whole when large numbers are obtained in different experiments and are averaged, a fairly consistent scheme results. Final judgment on this matter must, however, be withheld until the subject can be followed up by further experiments.

Another point which should be considered in this connection is the effect of differences in viability. In the case of P and M, used above as an illustration, the rudimentary winged flies are much less likely to develop than are the longs. Now if the viability of red and vermillion is different, then the longs do not give a fair measure of the linkage, and the rudimentaries, being present in such small numbers, do not even up the matter. It is probable that there is no serious error due to this cause except in the case of rudimentary crosses, since the two sides will tend to even up, unless one is very much less viable than the other, and this is true only in the case of rudimentary. It is worth noting that the only serious disagreements between observation and calculation occur in the case of rudimentary crosses (BM, and (CO)M). Certain data of Morgan's now in print, and further work already planned, will probably throw considerable light on the question of the position and behavior of this factor M.

SUMMARY

It has been found possible to arrange six sex-linked factors in *Drosophila* in a linear series, using the number of cross-overs per 100 cases as an index of the distance between any two factors. This scheme gives consistent results, in the main.

A source of error in predicting the strength of association between untried factors is found in double crossing over. The occurrence of this phenomenon is demonstrated, and it is shown not to occur as often as would be expected from a purely mathematical point of view, but the conditions governing its frequency are as yet not worked out.

These results are explained on the basis of Morgan's application of Janssens' chiasmatype hypothesis to associative inheritance. They form a new argument in favor of the chromosome view of inheritance, since they strongly indicate that the factors investigated are arranged in a linear series, at least mathematically.

November, 1912.

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A STUDY OF THE MALE GERM CELLS IN NOTONECTA

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TEN PLATES

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I. INTRODUCTION

Gilson, in his study of the spermatogenesis of the arthropods in 1885, passed over *Notonecta* with the remark, "Les phénomènes de la spermatogénèse y sont fort simples et présentent peu de particularités dignes d'être mentionnées" (op. cit., p. 123), adding that *N. glauca* possesses the longest and largest spermatozoa known. More recently, Pantel and Sinéty ('06) have published a copious memoir on "Les cellules de la lignée mâle chez le *Notonecta glauca* L.," and they, unlike Gilson, have found themselves "en présence d'un assez grand nombre d'images d'un aspect nouveau, parfois très inattendu ou même déconcertant" (op. cit., p. 90). Further work on this genus seemed to be warranted by the very peculiar appearances described by these authors, as well as by the acknowledged slight treatment of the maturation divisions in favor of the stages concerning the transformation into the spermatozoön. The problem was suggested to me by Prof. E. B. Wilson, to whom I wish to express my most sincere thanks for his valuable advice and criticism during the course of the investigation. This study is based on the three common American species, kindly identified by Mr. E. P. Van Duzee as *Notonecta undulata* (Say), *N. insulata* (Kirby) and *N. irrorata* (Uhler); the form used by the French authors was the European species, *N. glauca*. While the American species agree with *N. glauca* in presenting many very puzzling appearances, they differ from it in several important respects and also differ considerably among themselves. The two facts of main interest are, first, the presence of a karyosphere or body in which the chromatin is aggregated during the growth stages in all three species, as was noted also in *N. glauca* by Pantel and Sinéty; and secondly, the relation of the chromosome number to the species, a brief

summary of which has already been published (Browne '10). The present study deals only with the growth stages and maturation divisions, no attempt having been made to treat the later stages which have been elaborately worked out by Pantel and Sinéty.

II. MATERIAL AND TECHNIQUE

The material, consisting of the three species already mentioned, was collected during four summers at Woods Hole, Massachusetts. These species differ considerably from one another in size, in wing coloration and markings, and in other characters. *N. insulata* is the largest, with brown wings usually marked with two black bands. *N. irrorata* is slightly smaller, and its wings are black, mottled more or less with brown. *N. undulata*, the most common species, is considerably smaller than the other two and the wing color varies from a pure white to a white with one, two, or three black bands. In respect to germ cell production, there are two types. In *N. undulata*, all the stages of the spermatogenesis occur in the adult and even in the very young larva throughout the summer. In *N. irrorata* and *N. insulata*, during the greater part of the summer, the testis of the adult and late larva is filled with cells in the late growth stages, the younger cysts being empty except those at the very tip of the testis where a few spermatogonia occur. For only about a week during the summer are division stages found in these two species; after this the testis is filled with spermatids and spermatozoa. Pantel and Sinéty have noted the same slow evolution of the germ cells in *N. glauca*. Probably owing to this long period of growth, the cells of *N. irrorata* and *N. insulata* are larger than those of *N. undulata*. The great size of the cells coupled with the diagrammatic clearness of the spindle fibers and asters make the material exceptionally fine for the study of the maturation divisions. The French authors find it otherwise for *N. glauca*, stating that "La figure chromatique est d'un type malingre, dans les cinèses maturatives du *Notonecta*, et peu favorable à une analyse détaillée des phénomènes morphologiques" (op. cit., p. 136).

The only difficulty with my material has been the scarcity of spermatogonial divisions and early growth stages.

The testes are bifurcated coiled tubes lying on either side of the alimentary canal. They were dissected out in Ringer's solution and transferred at once to the fixing fluid. Flemming's strong fluid, Bouin, Carnoy, Gilson and corrosive sublimate were used with results that are favorable in the order named. Heidenhain's haematoxylin was used almost exclusively as a stain, though some safranin preparations were made. In order to demonstrate mitochondria, some of the testes were fixed with Benda's modification of Flemming's fluid, and these were subsequently treated with his mitochondrial stain of sulphuralizarinate of sodium and crystal violet, used according to his original method. The results of the fixed preparations have been controlled by observations of the living cells both with and without intra-vitam stains. By dragging the testis over a slide and mounting in a drop of Ringer's solution, very good results were obtained. The mitochondria and karyosphere may at once very clearly be seen; and after half an hour or so, the chromosomes in division stages come out very clearly. This is probably due to the fact that some change has taken place in the chromosomes, and it may be that they are not visible in the living state. Such would seem to be the case from the fact that constant observation of anaphase spindles failed to reveal any progression of the chromosomes toward the poles. In some cases it was possible to count the chromosomes in these preparations.

III. CHROMOSOMES

A. *Observations*

As pointed out in my preliminary paper ('10), the study of the chromosomes in *Notonecta* has proved of much interest from the fact that the change in number from species to species can here be attributed to the relations of a particular chromosome. Briefly the results are as follows. In all three species there is present an unequal XY-pair of chromosomes which divide separately in the first spermatocyte division but are united in the

second, thus making the total number of separate chromatin elements one greater in the first than in the second division. In *N. undulata*, there are 14 chromosomes in the first division, 13 in the second, including two small chromosomes. In *N. irrorata*, there are 13 in the first and 12 in the second, including only one small one. In *N. insulata* there are either 14 or 13 in the first, and 12 in the second; when there are 14 in the first, there are two small ones, when 13 there is only one free small one, but the other small one can often be detected attached to the largest chromosome. This species thus appears to be intermediate in respect to the chromosomes between *N. undulata* with a larger number, and *N. irrorata* with a smaller number.

1. *Notonecta undulata*. In *N. undulata*, the typical, and I am inclined to believe, the invariable, arrangement in the first spermatocyte division is a ring of 12 surrounding two very small chromosomes. This is shown in polar view in figures 1 and 2, and in side view of a spindle from two adjoining sections in figure 3 *A, B*. Very frequently side views present the appearance shown in figure 4 *B*, the two pairs of small chromosomes lying in a straight line, as though on the same spindle fiber (*A, B, C* are serial sections of the same spindle). This is probably due to the fact that they lie very close together and the smaller of the two pairs usually precedes the other in division. In the peripheral ring can be distinguished one chromosome larger than the rest, one very small one slightly larger than the central ones, and ten of intermediate and intergrading sizes.

In the second spermatocyte division, side views clearly show the presence of an unequal *XY*-pair (fig. 5 *B*). Since these chromosomes have divided separately in the first division, as is the case in many other Heteroptera, there should be one chromosome less in the equatorial plate of the second division. That there are 13 chromosomes in this division is shown in side view in figure 5 *A, B, C* (from the same spindle), and in polar view in figures 6 and 7. (In the latter figure the *X*-chromosome is seen at a lower focus). In this division, *X* and *Y* always take up their position in the center of the spindle, as they do in other Hemiptera. A rather interesting phenomenon occurs in *Notonecta*

with regard to the *XY*-pair. The two components frequently fail to conjugate, and lie in the second metaphase side by side, on separate spindle fibers (figs. 8, 9). A little later stage is shown in figure 10 *A*, where the small component is evidently going to one pole, the large one to the other; these are frequently connected at this time by an oblique fiber (fig. 11). The size relations of the chromosomes are evident from an inspection of figures 5, 6, 7, 9, 10. The two smallest chromosomes which were in the center in the first division are now in the peripheral ring. The third small one of the first division was apparently the *Y*-chromosome. The largest chromosome is again evident in the peripheral ring. The *X*-chromosome is one of the larger chromosomes, probably the third largest. The size relations come out very clearly in figure 12 *A, B*, which are sister anaphase groups from the same spindle. It is apparent from these groups that *X* is present in one of the resulting cells, *Y* in the other, and since these cells develop directly into the spermatids and thence into spermatozoa, the latter must be of two types in respect to the chromosome content.

In the spermatogonial groups, there are 26 chromosomes (figs. 13, 14), among which a largest and a smallest pair can easily be recognized. There are two pairs of very small ones, evidently corresponding to the two small bivalents in the center of the spindle in the first spermatocyte division. There are two very large chromosomes corresponding to the one large one in the haploid groups. Then there is a pair slightly smaller than these and another odd large one. This is evidently the *X*-chromosome, and *Y* is distinguishable as the fifth small chromosome which clearly has no mate of its own size.

2. *Notonecta irrorata*. In *N. irrorata*, the typical arrangement is a ring of 12 chromosomes surrounding one small one (figs. 15, 16). The second small chromosome which occurred inside the ring in *N. undulata* is here lacking. Serial sections of a spindle in side view showing the total number and the typical arrangement, are represented in figure 17 *A, B, C*. A few cases have been observed where the components of the central pair apparently fail to conjugate and lie on separate fibers in the metaphase; these are distinctly univalent in contrast to the other bivalents

(fig. 18). Their behavior resembles that of the components of the *XY*-pair in the second division, which may or may not conjugate before going to the poles; and it is also analogous to that of the *m*-chromosomes of the Coreidae which conjugate very late and do not fuse. As in *N. undulata*, one chromosome in the peripheral ring is larger than the others, and it is here in some cases longitudinally split (fig. 16). There are in this species two small chromosomes in the peripheral ring.

In the second division, the presence of an unequal *XY*-pair in the center of the spindle is evident from side views (fig. 19 *B*). Serial sections of a spindle in side view (fig. 19 *A, B, C*), and polar views (fig. 20), show that there are 12 chromosomes, in contrast to the 13 of *N. undulata*. Here too, the components of the *XY*-pair may fail to conjugate before the second division, and lie on separate spindle fibers in the center of the spindle (figs. 21 *B, 22*). It is evident that *X* and *Y* are less unequal in size than in *N. undulata*, *X* being comparatively smaller and *Y* larger. The largest chromosome is distinguishable among the others, and also the three small ones of the first division (the one in the center and the two peripheral ones). The fact that the result of this division will be two kinds of cells (ultimately spermatozoa) differing in chromatin content in respect to one chromosome is apparent from sister anaphase groups (fig. 23 *A, B*).

Only one clear spermatogonial group has been found (fig. 24); the number here is 24, including three pairs of small chromosomes, corresponding to the three small ones of the spermatocyte divisions; the largest pair corresponding to the large one of the haploid groups; two other large pairs, and one odd large one. This is doubtless the *X*-chromosome; the *Y*-chromosome is indistinguishable, but must be one of the smaller intermediate ones.

3. *Notonecta insulata*. *N. insulata* has proved an extremely interesting species from the fact that two distinct types of chromosome groups occur in the first division, in approximately equal numbers and side by side in the same cyst. One type has 14 chromosomes including two small ones in the center, like *N. undulata* (figs. 25, 26); the other type has 13 chromosomes, including only one small one in the center, like *N. irrorata* (figs. 27, 28).

The discrepancy in number was very perplexing until consecutive sections of complete spindles were examined as they appeared in side view. It was then discovered that in many cases the discrepancy is accounted for by the fact that *the second small chromosome which appears in the center in the 14-type is frequently found attached to the largest chromosome in the 13-type*. In figures 29-31 *A, B, C* are shown serial sections of three spindles which have only one small chromosome in the center, the other small one being attached to the large chromosome forming the compound chromosome *Ma* (macrochromosome + small autosome). Polar views of the compound chromosome are rather difficult to obtain owing to the small size of the smaller component. Such a view, from a spindle cut somewhat obliquely, is given in figure 32 where both components show very clearly. In figures 33, 34 *A, B, C*, are shown spindles of the other type, where both small chromosome pairs are in the center and the large chromosome is not compound. In over thirty cases where the chromosomes have been counted in consecutive sections in side view, the apparent 13-type has been found to be due to the attachment of the second small chromosome to the large chromosome. *It is always this particular chromosome, the largest one, with which the little one is associated*. In many cases, however, when only one small chromosome appears in the center, the compound character of the large one cannot be detected, the two components having probably fused beyond recognition. When there are two small ones in the center, there are 14 chromosomes, and the large chromosome is never compound.

Besides the two small chromosomes at the center of the spindle (or one in the center and the other attached to *M*) it is clear from an inspection of the figures that there is always another small chromosome in the peripheral ring. Attention may also be called to the fact that the largest chromosome is usually longitudinally split, as it is occasionally in *N. irrorata* (figs. 25-28, 32).

In the second division, the number of chromosomes is always 12, so far as I have observed (figs. 35-37). As in the other two species, an unequal *XY*-pair is here present in the center of the spindle (fig. 37 *A*), and the components are frequently found side by side, having apparently failed to conjugate (figs. 38, 39).

They are more nearly equal in size than in *N. undulata*, in this respect resembling those of *N. irrorata*. The invariable number 12 is accounted for on the assumption that the second small chromosome which in the first division is sometimes separate and sometimes associated with the large one, has fused with it in all cases before the second division. Additional evidence is given by the fact that in most cases two of the chromosomes are considerably smaller than the others, one of these corresponding to the small central one, and the other to the small peripheral one of the first division (figs. 35-39). The large chromosome, however, presents an unexpected appearance. It gives no evidence whatever of its real composition of two very unequal parts, but it appears in the metaphase as a large quadripartite chromosome, as though each part into which it divides were composed of two *equal* parts (p. 89). The longitudinal split which was very noticeable in the first division in polar view marks the division plane of the second division. In figure 40 *A, B* are shown two sister plates of an anaphase; the groups are identical except for the middle chromosome, and it is evident that on this account two kinds of cells are produced which give two kinds of spermatozoa.

Unfortunately no spermatogonial groups have been found of which a satisfactory count could be made. The expectation would be either 26 single chromosomes, or 24 including two compound ones.

4. *Notonecta glauca* (Pantel and Sinéty). According to the account of Pantel and Sinéty ('06), there are in *N. glauca* sometimes 12, sometimes 13 chromosomes in the first division. They state that they are unable to account for this difference, but they also say, "elle (la couronne équatoriale) comprend un anneau périphérique, plus une ou deux unités situées au centre" (op. cit., p. 139). No figures of polar views are given but it seems probable from the statement that the discrepancy is here due to the presence or absence of a second small chromosome in the center, as in the case of *N. insulata*. They do not state in the text the number present in the second division, but figure 12 chromosomes. The writers mention no unequal *XY*-pair in the second division.

If this pair is absent, *N. glauca* differs radically from the other three species, and it may be that this discrepancy is analogous to that found in *Metapodius* (Wilson '09 a) where in different individuals of the same species a *Y*-chromosome may be present or absent; or in the mosquitoes, where there is a typical unequal *XY*-pair in *Anopheles punctipennis*, while in two other genera, *Culex* and *Theobaldia*, the differential chromosomes are absent (Stevens '11). It is possible that the *X* and *Y* chromosomes are present in *N. glauca*, but are of practically equal size as in *Oncopeltus* and *Nezara hiliaris* (Wilson '11), and have been overlooked. Pantel and Sinéty call attention to the presence of an extra large chromosome, which they call the 'chromosome exceptionelle,' suggesting that it may be an accessory chromosome, but they are convinced that it participates in both divisions. This body seems quite similar in appearance and behavior to the large chromosome described in *N. insulata*. It is unfortunate that *N. glauca* cannot be brought into line with the three American species in regard to chromosome number, but the account of Pantel and Sinéty is inadequate to permit the attempt.

B. Discussion

1. *The relation of chromosome number to species.* It is doubtful whether every chromosome in the three species can be homologized individually, for the size relations are different in some respects. By comparing the spermatogonial groups of *N. undulata* and *N. irrorata* (figs. 13, 14, 24) it is evident that there are 5 large chromosomes in the former, and 7 large ones in the latter; and the *XY*-chromosomes are of different relative size in the three species. On the other hand, one largest chromosome can be traced throughout the history of all three species; likewise the smallest chromosome, not only by its size but especially by its position in the first spermatocyte division. We may also homologize the second small chromosome which is present in the first division of *N. undulata*, in the center of the group, with the one of similar size which is sometimes present in *N. insulata* in the same position. And since the steps in the process of fusion

can actually be observed in *N. insulata*, it seems reasonable to attribute its absence in *N. irrorata* to its permanent association with the largest chromosome. Representing the large chromosome, or macrochromosome by *M*, the two small autosomes by *a*, the unequal chromosomes by *X*, *Y*, and the larger autosomes by *A*, we may schematize the results as follows:¹

	PRODUCTS OF THE FIRST SPERMATOCYTE DIVISION	PRODUCTS OF THE SECOND SPERMATOCYTE DIVISION
<i>N. undulata</i>	$M + 9A + X + Y + a + a$ (14)	$\left\{ \begin{array}{l} M + 9A + X + a + a \quad (13) \quad \text{♀} \\ \text{and} \\ M + 9A + Y + a + a \quad (13) \quad \text{♂} \end{array} \right.$
	either	
<i>N. insulata</i>	$\left\{ \begin{array}{l} M + 9A + X + Y + a + a \quad (14) \\ \text{or} \\ Ma + 9A + X + Y + a \quad (13) \end{array} \right.$	$\left\{ \begin{array}{l} Ma + 9A + X + a \quad (12) \quad \text{♀} \\ \text{and} \\ Ma + 9A + Y + a \quad (12) \quad \text{♂} \end{array} \right.$
<i>N. irrorata</i>	$Ma + 9A + X + Y + a \quad (13)$	$\left\{ \begin{array}{l} Ma + 9A + X + a \quad (12) \quad \text{♀} \\ \text{and} \\ Ma + 9A + Y + a \quad (12) \quad \text{♂} \end{array} \right.$

The scheme shows the intermediate condition of *N. insulata* between *N. undulata* with a larger number of chromosomes and *N. irrorata* with a smaller number. The large and small chromosomes in *N. undulata* are always separate, in *N. insulata* sometimes separate and sometimes associated, and in *N. irrorata* are presumably always associated. This may represent a progressive (or regressive) series, or the three forms may represent different modifications of a single original type.

The somatic characters do not afford decisive evidence concerning these three possibilities, although the wing color fits in with the view that *N. insulata* is an intermediate species. By substituting brown pigment for the white of *N. undulata*, the wing coloring and pattern of *N. insulata* is obtained; further, by substituting for this brown pigment, black, but leaving some of the brown as mottling, the wing pattern of *N. irrorata* is obtained. On the other hand, *N. irrorata* is intermediate in size between the other two, and *N. undulata* is intermediate in respect to the distance

¹ This scheme is identical with that published in my preliminary paper ('10) except that *X*, *Y* have been substituted for *I*, *i*.

between the eyes. It may be that the wing color is directly correlated with the fusion and separation of the two chromosomes, and that the other somatic differences are connected with other chromosomes, which, as I have stated, differ in size in the different species.

In the Acrididae, McClung ('05) has found that a particular genus, *Hesperotettix*, is distinguished from others of the family by a special arrangement of the chromosomes, by which the accessory is always associated with another chromosome forming a multiple element. He concludes that this arrangement "is genetically connected with the subsequently appearing characters" (op. cit. p. 326). This correlation of a multiple chromosome element and a generic difference in the Acrididae is directly comparable to the correlation of a multiple chromosome element and a specific difference in *Notonecta*.

The correlation of a definite number of chromosomes with a particular species is a well established fact throughout the animal and plant kingdoms, and is admitted by practically all cytologists, with only a few exceptions. In several cases, however, it has been shown that the number is constant for the individual, but differs for different individuals. This is sometimes due to the presence of 'supernumerary' chromosomes, as in *Metapodius*, *Banasa calva*, *Diabrotica* and *Ceuthophilus* (Wilson '09 a, Stevens '12 a, b), and sometimes to the fact that two types of chromosome groups occur within the species, one with twice the number of the other, as in the well known cases of *Ascaris megalocephala*, *Echinus microtuberculatus* and *Helix pomatia*, and as in *Artemia salina*, as recently pointed out by Artom ('11). *Cyclops viridis* is apparently a species in which different numbers occur in different varieties (Chambers '12). With these and possibly a few other exceptions, the number of chromosomes is a specific characteristic, although occasional fluctuations may occur.

Further, there are many cases where closely related species have the same number of chromosomes. For example, five species of *Euschistus* have the same number (Montgomery, Wilson); four species of *Sagitta* (Stevens '10), and three species of *Ceresa* (Boring '07). In other cases, related species differ only slightly

in number. For example, three species of *Podisus* have the diploid number of 16, two species 14 (Montgomery, Wilson). In this category belong the three species of *Notonecta*. But much wider differences in related species may occur, as for example, in two closely similar species of *Banasa*, of which *B. dimidiata* has 16 chromosomes, *B. calva* has 26 (Wilson '09b). So in *Thyanta*, in which a distinction between two species has just recently been rediscovered by Barber, two types of chromosome groups occur, 27-28 in *T. calceata*, and 16 in *T. custator*. Among the phylloxerans, there is considerable variation; four species have 6 chromosomes in the diploid groups, one species has 8, one 12, and one 22 (Morgan '09). Similarly in the aphids, the haploid number ranges from 3 in the willow aphid to 16 in the maple aphid (Stevens '06). Likewise Braun ('09) found in fifteen species of *Cyclops* a wide range of number, from 6 to 22, although several species have the same number. In the *Oenotheras*, mutants have been found with 14, 15, 21 and 28 chromosomes (Lutz '12).

When we come to groups less closely related than species, marked differences in the chromosome number frequently occur. In the family Jassidae, the diploid number varies from 15 to 23 in different genera; in the Cercopidae, from 15 to 27; in the Membracidae, from 17 to 21 (Boring '07). In ten genera of the Chrysomelidae, there is a range from 16 to 36 (Stevens); in the Coreidae, from 13 to 27 (Montgomery, Wilson). These are a few of the many cases of divergence within a family. There are on the other hand, a few cases where a constancy obtains throughout as wide a group as a family, as for example, in ten genera of the orthopteran family, Acrididae (McClung '05, et al.), and in four genera of the opisthobranch mollusks. The constancy in number goes still further in some of the Amphibia, where all the urodeles, so far as examined, apparently have 24 chromosomes in the diploid groups.

It is therefore evident that while in some cases the chromosome number is the same for members of rather a large group, it is not necessarily the same for even very closely related forms. It is true in general, however, that closely related forms have the same

or very nearly the same number of chromosomes. This fact has led Montgomery and McClung to the view that the number and arrangement of chromosomes should be considered as an important character in taxonomy. More recently, McClung ('08) has expressed this view forcibly in his paper on "Cytology and Taxonomy." It is of interest in this connection to find that in the *Oenotheras*, according to Lutz ('12), all individuals having a given type of vegetative character have the same number of somatic chromosomes, irrespective of the origin of these individuals, whether hybrid or mutant.

That one method by which a change in the chromosome number has taken place is by the fusion or separation of particular chromosomes seems highly probable from the evidence given by *Notonecta*, where we have all the stages in the process in the three species. Such a process may also be indicated by the *d*-chromosome in *Nezara* (Wilson '11). A somewhat similar idea was put forth by Montgomery ('01) before the relation of the *X*-chromosome with sex had been established, to explain the occurrence of an odd number of chromosomes in the spermatogonial groups of some of the Hemiptera; the odd number representing, he believed, a transition stage between two even numbers. A change in number by a process of fusion has been advocated by McClung ('05) in regard to the multiple chromosomes in the Orthoptera. A change in number by a process of splitting has been advocated by Payne ('09) in the case of the multiple *X*-element in the reduvioids, and this may likewise apply to that of many other forms, such as *Phylloxera*, *Syromastes* or *Ascaris lumbricoides*, as has been indicated by Wilson ('11). A second probable method of change is by a process of progressive reduction and final disappearance of particular chromosomes, as was originally suggested by Paulmier ('99) in the case of the small *m*-chromosomes of the Coreidae, and later by Wilson in the case of the *Y*-chromosome.

These two methods will account for gradual and slight changes in the chromosome number. Such wide variations as occur in closely related species, e.g., in *Banasa*, *Thyanta*, and the phylloxerans, must be accounted for in some other way. Wilson ('11)

suggests that some sudden mutation has taken place, involving a new segregation of the nuclear material, and causing a change in number and size relations of the chromosomes, but not in their essential quality.

A fourth method by which either a slight or a radical change in the chromosome number might take place is by an abnormality occurring in mitosis, as has been suggested by several authors, either by an unequal distribution of the chromosomes to the daughter cells, or by an arrest of cell division after a division of the chromosomes. The former abnormality has actually been observed in the case of *Metapodius* (Wilson '09 a), and the *Oenotheras* (Gates '08, et al.). The possibility of the occurrence of the second abnormality is shown by the experiments of Gerassimow ('01) on *Spirogyra*, of Němec ('04) on *Pisum* and of Boveri ('05) on sea-urchin eggs, in which a monaster was produced instead of an amphiaster, the chromosomes dividing but not the nucleus, and the double number of chromosomes remaining in subsequent divisions. To this cause has been attributed the occurrence of triploid and tetraploid mutants in *Oenothera* (Gates '09, Lutz '12), and it seems probable that many of the cases of a double number of chromosomes occurring in closely related forms of some animals (e.g., *Ascaris megalocephala*), and many plants have been brought about in this way.

2. *Temporary association and separation of chromosomes.* The condition of temporary association and separation of particular chromosomes which occurs in *N. insulata* is of especial interest in comparison with other forms. In the first place, there are cases where the union and separation concerns the sex-chromosomes only. In these cases the X-element may consist of two or more components—in *Acholla* (Payne '09) and *Ascaris lumbricoides* (Edwards '10) as many as five—that appear as separate chromosomes in the diploid nuclei but become associated in the spermatocyte divisions and behave as a single accessory.

In a second category may be placed those forms where there is a temporary or permanent association of the sex chromosomes with other chromosomes. Sinéty ('01) was the first to describe a case of this sort in the phasmids *Menexenus* and *Leptynia*.

Here the accessory becomes attached to another chromosome in the first division, and goes over with it to one pole. McClung ('05) describes a similar relation for the acridian *Hesperotettix* and the locustid *Anabrus*. In *Hesperotettix* he found that it is always the largest chromosome with which the accessory is associated. In *Mermeria*, another acridian, a similar multiple element becomes further associated with another tetrad, and in division this complex acts as a single bivalent, with the anomalous result that entire tetrads pass to one pole. More recently, Boring ('09), Boveri ('09) and Edwards ('10) have found in the case of *Ascaris megalocephala* that the accessory may be free or may be indistinguishably united with another chromosome. Stevens ('11) similarly finds in one of the mosquitoes a close union of X and Y with a pair of autosomes in the spermatocyte divisions, while in the spermatogonia they may or may not be closely united with them.

In a third category we may place the form *N. insulata* where there is a temporary association and separation of two ordinary chromosomes (autosomes). That this association has some significance can scarcely be doubted when we consider that it is always two particular chromosomes that are united. If the union of the two chromosomes is the primitive condition, then the secondary separation might mean that certain characters are being segregated from other characters. If the separation is primary, the fusion of the chromosomes might mean that a certain series of characters which were entirely independent of another series have become linked with them. It is possible that just as the association of a sex-chromosome and an autosome may serve as a morphological basis for sex-linked inheritance as pointed out by Wilson ('11), the association of two autosomes may give the morphological basis for the cases of coupling that have recently been made known in experimental work. For example, Bateson and Punnett ('11) find that in the sweet pea, blue color and long pollen are usually combined, red color and round pollen, etc.; and in *Primula*, according to Gregory ('11), magenta color is coupled with short style. In *Drosophila* also, a linkage of a color and a wing factor has been found by Morgan and Lynch

(12). In order that the linkage may take place, however, in *N. insulata*, we must assume that a particular chromosome of one pair always associates with a particular one of the other pair and never with its mate. When these two chromosomes are permanently associated, as is probably the case in *N. irrorata*, one chromosome might serve as the basis of the linked characters.

3. *The XY-pair.* The observations on *Notonecta* add nothing new to the main facts in regard to the XY-chromosomes. The difference in size between the two components is much more marked in *N. undulata* than in the other two species; similar differences between related species have been found in *Nezara*, *Euchistus* and other Hemiptera. The only departure from the usual behavior of the XY-pair is in the failure of the two components to conjugate. Usually in the Hemiptera the components come together in the prophase of the second division, in contrast to all the other chromosomes which have paired before the first division. In *Notonecta*, however, in all three species this pairing frequently does not take place, and the two components of the XY-pair lie side by side in the metaphase of the second division and pass to opposite poles. A similar condition has been seen by Montgomery ('10) in *Euchistus*, but here it is apparently very exceptional for he found it only in one case out of 672. As to the time of conjugation of chromosome pairs, there is a graded series. The autosomes conjugate in the general synaptic period; the *m*-chromosomes undergo a late synapsis in the prophases of the first division; the XY-chromosomes in most Hemiptera do not finally conjugate till the end of the first division; the XY-chromosomes of *Notonecta* frequently do not ever form a definitive dyad. It is of interest, from the point of view of the mechanics of division, to find that a linear arrangement of the components of a chromosome pair is not necessary for their distribution to the opposite poles of the spindle.

IV. KARYOSPHERE²A. *Notonecta insulata*

1. *Formation of karyosphere.* The growth stages of the primary spermatocyte are probably separated by a considerable interval from the last spermatogonial telophase. In the earliest spermatocytes observed (fig. 41), the chromatin is massed in a single large body, the karyosphere, and thin strands of linin are scattered through the nucleus. The first change to take place is the accumulation of chromatin on the linin threads (fig. 42). Although the source of this chromatin cannot be definitely ascertained, it seems most likely, from a study of many of these nuclei, that it comes from the karyosphere following the course of the linin strands and tending to aggregate at particular points. The threads from the karyosphere are more or less twisted, and show a distinct radial arrangement. The tendency of the chromatin to aggregate in clumps becomes more marked until the nucleus is filled with small chromatin masses connected with each other and with the karyosphere by thin deeply staining strands (fig. 43). During this process the chromatin masses are frequently approximated in pairs (figs. 42, 43). This fact suggests that the masses represent chromosomes which are conjugating. Although the evidence is not conclusive that synapsis takes place at this time, the whole process of the formation of these chromatin masses seems unintelligible otherwise. The number of the chromatin masses varies considerably in nuclei of the same cyst; the maximum number is however greater than the reduced number of chromosomes, although probably not as large as the somatic number. This is easily accounted for on the assumption that the pairing of different bodies takes place at different times, as seems

² The term 'karyosphere' is used in this paper in the sense in which Blackman ('03) first used it to denote a structure consisting of chromatin and other substances, such as linin and karyolymph. It is thus a broader term than karyosome or net-knot or chromosome-nucleolus which is usually applied to a mass of pure chromatin. 'Karyosphere' is practically identical with Carnoy's 'nucléole-noyau,' or miniature nucleus; it is however difficult to determine the presence of a membrane as is required by his definition. Although it is impossible to tell at all stages whether accessory material is present with the chromatin, the term karyosphere will be used throughout the discussion.

to be the case. This process in *N. insulata* is somewhat similar to that described by Arnold ('08) in *Hydrophilus*, and by Davis ('08) in some of the Orthoptera. The masses are perhaps comparable with those described in many plant cells as 'prochromosomes.'³

After the stage of the scattered chromatin masses in *N. insulata*, a process of absorption sets in. The masses gradually decrease in size and the connecting strands become thicker, especially in the region of the karyosphere (fig. 44). By an absorption of all the chromatin masses, a spireme of approximately uniform thickness is formed which is irregularly coiled about in the nuclear cavity (fig. 45). The spireme is not formed here by an unraveling process as described by Janssens, Davis and others, but by a uniform distribution of the chromatin material along the connecting strands. A somewhat similar formation of the leptotene spireme has been described by Gérard ('09) in *Stenobothrus*.

The spireme now becomes arranged in loops which are more or less oriented toward the karyosphere and may connect with it at their apices (fig. 46). The karyosphere seems to act as a center of activity like the chromoplast of Eisen and Janssens. Later the loops take up a position on the nuclear wall, receding from the karyosphere which remains in the interior (fig. 47). The loops then become somewhat irregular, coiled and thicker; their staining capacity gradually diminishes until in faintly stained preparations only the karyosphere and a few scattered remnants of the loops take the chromatin stain (figs. 48-50). This change can be readily appreciated by comparing these three figures, which are from the same slide. The nuclear cavity is, however, filled with a flocculent reticulum, which is quite faint in lightly stained preparations, but is very noticeable and takes a deep chromatin stain in preparations that are less extracted.

The foregoing facts in *Notonecta* are extremely perplexing. Since the spireme is formed from the scattered masses, it must apparently at one time contain the essential elements of the chromosomes. A transfer of these elements into the karyosphere

³ These are evidently similar to the 'massive bodies,' recently described in other insects by Wilson ('12), as occurring in Stage b.

may be afforded in two ways; by a flow along the whole length of the spireme and (for a brief period) by the connection of the curves of the loops with the karyosphere. After the loops become disconnected and oriented, they give somewhat the appearance of the 'bouquet,' though this is never so regular or clearly marked as in *Batrachoseps*, *Tomopteris*, etc. The polarized loops of the bouquet stage are in other forms the forerunners of the chromosomes. This may also be the case in *Notonecta*, but if this be so, the conclusion seems unavoidable that the fundamental material must subsequently return to the karyosphere, for, as will be shown, the chromosomes later arise directly from the latter. It is possible that this material flows back into the karyosphere along the faintly staining threads that can usually be traced from the loops; but it seems more probable that after the loops are disconnected, their substance does not enter the karyosphere. This conclusion is based on the fact that the loops withdraw from the karyosphere, and that some of the more remote threads keep the chromatin stain after the ones near the karyosphere have lost it. If this be so, considerable ground is given for the view that there are here two kinds of chromatin, corresponding with those designated by Lubosch ('02) as trophochromatin and idiochromatin. The chromatin, which is later to form the chromosomes is transferred to the karyosphere in one or both of the two ways suggested, while the rest of the chromatin becomes disconnected from the karyosphere and is represented by the flaky reticulum in the nuclear cavity.⁴ In the case of *N. glauca*, Pantel and Sinéty have likewise concluded that the material in their 'moniliform cords' becomes achromatic and is spread through the nuclear cavity, taking no part in the chromosome formation. This material has no doubt a metabolic function during the enormous growth of the spermatocyte. From a comparison of figures 51 and 53 drawn to the same scale, it is evident that the surface of an equatorial plane of the full grown spermatocyte nucleus is approximately five times that of the youngest one, which means that its

⁴ The paper of Vajdovsky ('12), containing very interesting observations, bearing on this and other subjects here treated, unfortunately came to my notice too late for his results to be incorporated in this article.

volume is nearly twelve times as great. About half of the increase in size takes place before the disappearance of the spireme, the other half after the karyosphere is fully formed (figs. 51, 52, 53). The growth of the spermatocyte in *Notonecta* is so great as to be comparable with that of an oocyte (as was noted also by Pantel and Sinéty), and it must involve a similar metabolic activity. It is well known that in the eggs of many forms, some of the chromatin is eliminated during the growth or maturation divisions; this is probably correlated with the metabolism of the cell. The diminution of the chromatin by a casting off of the ends of the chromosomes in *Ascaris megalocephala* and *A. lumbricoides* is probably of a similar nature. The ring in *Dytiscus* (Giardina '01) which during four divisions passes to only one of the resulting cells, i.e., the oocyte, has likewise been interpreted by Boveri ('04) and Goldschmidt ('04) as representing chromatin that is concerned in the nutrition of the cell. For a comprehensive account maintaining the existence of two kinds of chromatin, basichromatin and oxychromatin, see Stauffacher ('10).

2. *Description of karyosphere.* The appearance of the karyosphere varies considerably with the stage of growth, with different fixing fluids and stains and with the amount of extraction. In many preparations, especially those stained deeply with haematoxylin, no structure is evident; it is merely a round or approximately round mass of vesicular appearance (fig. 54 A). In other preparations an irregular contour and difference in staining capacity in different regions gives it a spongy appearance (figs. 54 B, 55). In haematoxylin preparations well extracted and in safranin preparations the structure is quite definite. The karyosphere consists apparently of dense, compact bodies of varying size embedded in a less dense matrix (figs. 53 A, 54 C, D), the former being probably the chromatin proper. When crowded these bodies give somewhat the appearance of a continuous spireme closely convoluted (fig. 53 A). In the younger stages, the karyosphere tends to have a vesicular appearance, and later the spongy or granular structure is more evident. In the living material, the differentiation of two sorts of material in the karyosphere is perfectly evident, the denser substance taking the form of com-

pact masses, either isolated or continuous, embedded in a less dense substance (fig. 56). In this condition the karyosphere persists during the whole growth period.

3. *Dissolution of karyosphere.* Just prior to the formation of the aster, the karyosphere tends to assume a more definite outline appearing in lightly stained preparations as a more or less spherical body in which darkly staining bodies are embedded. Figure 57 is from the same slide as figure 58, the former being an earlier stage. After the formation of the aster, the chromatin masses leave the karyosphere as compact bodies, either irregular in shape or threadlike (figs. 59-61). It is perfectly evident that the karyosphere is breaking up into its two constituents; the darkly staining chromatin bodies are passing out of the karyosphere, leaving the less dense, paler material which becomes rounded and now appears as a typical plasmasome. As the masses leave the plasmasome, they quickly proceed to the periphery of the nucleus where they take on the form of double threads, as will be described later. In figure 60 the plasmasome is entirely free of chromatin, staining a pale gray; some of the chromosomes are seen along the nuclear margin. The mass of chromatin that has just left the plasmasome, is the large chromosome, *M*, which has been mentioned previously as one element of the compound chromosome typical for this species. The plasmasome has usually one or several small vacuoles in the interior. The body gradually decreases in size and disappears in the late prophase.

B. Notonecta undulata

1. *Formation of karyosphere.* In the earliest spermatocyte, a small karyosphere is present, and the rest of the nuclear cavity is filled with a reticulum of linin (fig. 62). The reticulum increases slightly in staining capacity and takes on the appearance of a very thin spireme, often twisted in spirals; a leader is usually to be seen running from the karyosphere (fig. 63). This is undoubtedly the leptotene stage. The spireme becomes more heavily staining and tends to contract to one side of the nucleus; the threads are still very thin, and in some cases appear

to run parallel in pairs (fig. 64). This is probably a synizesis stage and it is likely that a conjugation of the parallel threads takes place, although this cannot be conclusively demonstrated. In the next stage, the spireme is somewhat thicker and becomes arranged in loops, oriented toward the karyosphere (fig. 65). The loops next become attached to the karyosphere at their apices; this gives the appearance of arms radiating out from the karyosphere (fig. 66), and affords an opportunity for the transfer of material from the loops to the karyosphere. Very frequently the threads give the appearance of being longitudinally split. The loops now recede from the karyosphere and become arranged on the nuclear wall while the karyosphere remains in the interior (fig. 67). The loops gradually disappear, and the nucleus is filled with an irregular reticulum which is appreciable when the staining is dark, but pale when the stain is more extracted, in contrast to the dark karyosphere.

The early history of *N. undulata* thus differs markedly from that of *N. insulata*. There is no formation of scattered chromatin masses, but instead a leptotene stage which is followed by a synizesis or contraction, after which a spireme is present which is quite similar to that of *N. insulata*. The fate of the material in the spireme offers the same difficulties as in *N. insulata*, but it seems probable that here too some chromatin passes into the karyosphere, and other chromatin is segregated out and furnishes the material in the nuclear cavity.

2. *Description and dissolution of karyosphere.* The karyosphere of *N. undulata* appears very much as it does in *N. insulata*; it tends to be vesicular in the early stages, especially in darkly stained preparations, but later appears granular or rope-like (figs. 68, 69). At the approach of the prophase the karyosphere becomes broken up into a variable number of small round bodies, giving the appearance of a mass of marbles (fig. 70). The mass loosens and from it project one or two longitudinally split threads (figs. 71, 72). That the thread may be formed directly from the balls which become arranged in pairs is evident from figure 72. As the double threads form, they go to the nuclear wall where they become the diffuse prophase chromosomes which will be

described later. In figure 73, some of the chromosomes already lie at the periphery while others are still being transformed from balls into threads in the interior. From this process it seems clear that the material of one particular chromosome may be broken up into more or less isolated bodies, which later arrange themselves and fuse together to give rise to a continuous structure. As the last chromosomes form, some of the substance of the original karyosphere is left behind as a plasmasome. This body must apparently be formed from material that was in the balls which segregates out in the process of thread formation. The plasmasome is not colorless as it is in *N. insulata*, but remains dark even in well extracted preparations, probably owing to the fact that some of the chromatin is left behind. The plasmasome has a vesicular appearance and is frequently vacuolated. It gradually decreases in size and disappears in the late prophase.

C. Notonecta irrorata

The earliest stage occurring in my material is represented in figure 74. This shows the presence of a looped spireme on the nuclear wall, more or less oriented toward the karyosphere. The spireme gradually disappears as in the other two species, probably giving rise to the flaky material in the nuclear cavity. The karyosphere at this stage is distinctly vesicular. Just before the aster forms, however, it breaks up into a mass of balls of an inconstant number which form threads, very much as described for *N. undulata* (figs. 75, 76, *A*, *B*). A plasmasome is formed during this process which sometimes takes a heavy chromatin stain (fig. 77), and sometimes appears grey and vacuolated; it gradually decreases in size and disappears in the late prophase.

D. Conclusions and comparisons

A karyosphere is apparently present in the three species of *Notonecta* which I have examined, throughout the entire history of the spermatocytes. In the very early stages and in the later stages, this is the only body in the nucleus that takes a deep chromatin stain, but there is an intervening stage when a chromatic spireme is present. It would appear that the chromatic

material comes from the karyosphere, and that later, at least that part of it which contains the essential elements, returns to the karyosphere. This flow of material back and forth from the karyosphere seems highly remarkable, and is probably concerned with a rearrangement of the chromatin particles, for which a particular structure (i.e., the spireme) is necessary. It is unfortunate that I have not been able to determine more definitely how the definitive karyosphere is formed. In *N. glauca*, according to Pantel and Sinéty, a pale nucleolus is present in the early stages and the chromatic material from the 'moniliform cords' condenses around it, a process similar to that described for some of the myriapods (Blackman '05 b, '07) and the dragon fly (McGill '06). In other myriapods, the accessory chromosome is the center around which the other chromosomes are deposited to form the karyosphere (Blackman '05 a, '07, Medes '05).

In *Notonecta*, the chromatin remains massed together in the karyosphere, in an apparently inactive state during a long growth period. It is usually possible at this time to distinguish the chromatin material as distinct bodies, not necessarily the individual chromosomes, embedded in a less dense (plasmasome) material. Such an intimate association of plasmasome and chromatin material, where the latter is recognizable as distinct bodies, has been described in some of the myriapods (Blackman, Medes), and in the case of the XY-chromosomes in some of the reduvioids (Payne '09) and in certain Coleoptera and Diptera (Stevens). In some cases, e.g., in *Scolopendra heros* (Blackman '05 a) and in *Hydrophilus* (Arnold '08), there is apparently no plasmasome material associated with the karyosphere. The distinction of two sorts of material is extremely apparent in *N. insulata* in the early prophase, when the chromatin leaves the karyosphere as compact masses, and the remaining material becomes a typical pale plasmasome. In *N. irrorata* and *N. undulata*, and apparently also in *N. glauca*, the dissolution of the karyosphere takes place a little differently, by breaking up at once into a number of separate elements. In either case, there can be no doubt that the material which forms the chromosomes comes from the karyosphere. The events described for *Notonecta* do not seem to me at variance with the hypothesis of the genetic continuity of the

chromosomes; it seems, on the contrary, altogether reasonable to suppose that the essential chromosome elements retain their identity throughout the entire process.

V. PROPHASES

A. General description

1. *Notonecta insulata*. It is of interest to trace the history of the chromatin from the dissolution of the karyosphere until the formation of the definitive chromosomes. After the irregular masses of chromatin have left the plasmasome, they pass from the interior of the cell to the nuclear membrane; and here the chromosomes pass through a diffuse stage before assuming their final form. At first they appear on the nuclear wall as longitudinally split rods, long, thin and somewhat curved (fig. 78); the rods are apparently made up of a linear series of granules (chromomeres) which give them an irregular contour. The usual prophase figures, rings, crosses, etc., are formed from the longitudinally split threads (fig. 79); they will be described in detail later. By a process of condensation are formed the definitive chromosomes which are typically dyad-like in appearance; their tetrad nature cannot be made out unless they lie in a favorable position and are very critically observed (fig. 80). During these stages, the chromosomes have remained close against the nuclear membrane, and it is from this position that they are drawn on to the spindle in the late prophase. In figure 81 they are seen irregularly arranged on the spindle, prior to their final grouping around the equator. Attention may be called to the fact that frequently in the late prophase, the small chromosome is found attached to the large one, forming the compound chromosome, to which reference has been made in an earlier part of the paper (figs. 80, 81).

2. *Notonecta irrorata*. The history for this species is practically the same. The thin longitudinally split rods (fig. 82) on the nuclear wall give rise to rings, crosses, etc. (fig. 83). While still on the nuclear wall, they condense into the definitive chromosomes, which later become irregularly arranged on the spindle (fig. 84).

3. *Notonecta undulata*. In this species also, the first indication of the final chromosomes is the presence on the nuclear wall of thin, more or less coiled threads (fig. 85). The prophase figures which these form are quite different, however, from those of the other two species. At first these have a very vague, spongy appearance and are coarsely granular (fig. 86). By a process of condensation, they become more compact and more definite in outline (fig. 87). The figures are quite irregular in shape, but in general consist of two bars, diverging or united at one or both ends. While in this stage, the nuclear membrane breaks down and the spindle fibers begin to form. The chromosomes are still quite irregular in shape after the spindle is fully formed (fig. 88), and do not assume their definitive form until the full metaphase.

B. Detailed description

1. *Notonecta insulata*. a. The ring. The *M*-chromosome is usually the last one out of the plasmasome, and is therefore in the interior of the nucleus at the time that all the other chromosomes are in a diffuse condition on the nuclear wall (fig. 60). Owing to this fact and also to its greater size, its history can be traced throughout the prophase and also during the first and second maturation divisions. Whereas the other chromosomes come out of the plasmasome in more or less irregular masses, the *M*-chromosome has the form of two rods, somewhat coiled about each other, but in general taking the same direction (fig. 89 *A-D*). The two rods untwist, and open out in the middle, usually becoming or remaining united at the ends (fig. 90 *A-D*). By opening out still more, a small ring is formed (fig. 91 *A-D*); frequently at this stage and occasionally earlier, a longitudinal split is present, in one or both half rings. If we term the original line of separation between the two rods a longitudinal split, this is the second longitudinal split. By this time, the *M*-chromosome has reached the nuclear wall, and at once a process of expansion sets in. The ring opens out until the enclosed space becomes relatively very large and the ring itself correspondingly thin (fig. 92 *A, B*). It

is usually broken at this stage into two half rings, each one corresponding to one of the original rods; and each half ring is clearly longitudinally split. The two bars of each half ring frequently intertwine, thus making a quite remarkable figure (fig. 92 *A*). There seems to be no fusion at the points of crossing and no connection of the elements of the two half rings inter se, so that the figure lends no support to Janssens' ('09) chiasmotype theory. After the stage of maximal expansion, a process of condensation sets in during which the enclosed space becomes smaller and the bars thicker; the first stage of the process is shown in figure 93 *A-F*. The second longitudinal split has become so pronounced that it entirely separates each half ring into two distinct elements. The quadripartite nature of the ring is especially noticeable at the juncture of the two half rings, for here the longitudinal bars diverge considerably. In the very late prophase, the *M*-chromosome appears as shown in figure 94 *A-D*; the space enclosed in the ring has become very much reduced, and the second longitudinal split is still in evidence. The chromosome becomes arranged on the spindle with its first longitudinal split in the plane of the equator and its second longitudinal split in the plane of the spindle axis. In a side view of the chromosome on a metaphase spindle, therefore, the second longitudinal split is not visible, since it lies in the plane of the paper (fig. 95 *A*, also figs. 29 *C*, 30 *C*, 31 *C*, 33 *C*, 34 *A*). If however one obtains an end view of the chromosome as it lies on the periphery of the spindle, i.e., so that the place of union of the four elements is in the line of vision, the second longitudinal split is clearly seen at right angles to the first (fig. 95 *B*). Also, in polar view of a metaphase plate, the second longitudinal split is so clearly marked, that the *M*-chromosome seems to consist of two distinct parts (fig. 95 *C*; also figs. 25-28, 32). The first division plane passes through the first longitudinal split. The second longitudinal split remains during the anaphase; figure 96 *A* is a view of the chromosome cut obliquely so that one of the components is at a higher level than the other; figure 96 *B* shows the compound chromosome *Ma* in end view. Figure 97 is a late anaphase showing the bipartite nature of the *M*-chromosome. This is also evident in

figure 98 where the chromosomes are being pulled on to the second spindle immediately after the completion of the first division. On the spindle, the *M*-chromosome lies so that the longitudinal split is in the plane of the equator; the split therefore marks the line of division (fig. 99). The daughter groups in the late anaphase of the second division are shown in figure 100. The *M*-chromosome in the second division has rather a peculiar form for this stage. In the metaphase it looks like a tetrad, and after division like a dyad, but this bipartite appearance of the single element has probably no significance. The four chromatids have been distinct since long before the first division and each has retained much the same form throughout its history; this form happens to be a dyad-like structure.

To sum up: the *M*-chromosome starts as a double rod which opens out to form a ring; a second longitudinal split appears. In the first division, it divides along the first longitudinal split into what were two half rings. In the second division each part divides along the second longitudinal split which has remained since it was formed.

It is not only the *M*-chromosome that forms a ring, but the next largest chromosome goes through a similar history, as far as it can be traced. Starting with the open ring which is longitudinally split (fig. 101 *A*), it passes through stages in condensation, exactly parallel with those of *M* (fig. 101 *B-D*). In the metaphase of the first division, in side view, the longitudinal split is not visible since it lies in the plane of the paper (fig. 101 *E*), but in polar view it divides the chromosome in two halves (fig. 101 *F*). The line of division coincides with the plane between the two half rings. In figure 102 is shown a late anaphase group in which one may distinguish the largest and the next largest chromosomes, both longitudinally split. The split in both cases marks the line of separation for the second division.

In addition to the two large rings, there is a small ring in the prophase, which also has a longitudinal split (fig. 103 *A, B*). Its history has not been traced.

b. The cross. At the time that the *M*-chromosome is leaving the plasmasome, the other chromosomes are on the nuclear wall

in the form of thin double rods (fig. 60). The genesis of the cross from these bodies is as follows. The two segments open out into a *V*, each arm of which becomes longitudinally split (fig. 104 *A*). The arms of the *V* open out still further so as to form a double straight rod, the original space between the arms (i.e., the first longitudinal split) being represented only by the small opening in the middle of the two bars (fig. 104 *B*). There are evidently two methods by which a tetrad may be formed from this figure. The double bars may condense, while the connection around the central opening becomes very thin (fig. 104 *C, D*), or the connection around the central opening may become pulled out transversely, so as to form the cross-bars of a typical cross (fig. 104 *E, F*). In this case, half of each long arm and half of each short arm condense to form one element of the tetrad (fig. 104 *G*). The end result is the same in either case, a tetrad is formed in which the original longitudinal split is represented by the division line through the short axis and the second longitudinal split by the line through the long axis. In the metaphase, the tetrad lies with its long axis parallel with the spindle and its short axis in the plane of the equator. The first division therefore separates the two components of the original double rod. The vertical split is usually rather difficult to make out with certainty in the metaphase but in some cases is quite clear (fig. 104 *H*). This split becomes very distinct in the anaphase, and marks the line of separation of the second division (fig. 104 *I*).

c. The double rod. By a process of condensation, the original double filament forms a thick double rod, the two components of which lie parallel (fig. 105 *A, B*). These become united at one end, and straighten out to form a dyad (fig. 105 *C, D*). There is no clear evidence of the presence of a second longitudinal split. In the metaphase, the chromosome lies with its original longitudinal split in the plane of the equator, so that the first division separates the two components of the original double rod.

d. *XY*-pair. In figure 106 *A* is shown a diffuse cross which differs from the ordinary cross described above only in the fact that its longitudinal bars are unequal. It is possible, of course, that this is an ordinary cross of which part of one bar has been

cut off in the section. The same may be said of figure 106 *B* where the cross is more condensed. But the probability that these represent stages in the history of the *XY*-pair is suggested by the occurrence of an unequal tetrad in the late prophase (fig. 106 *C, D*). The two small components have, in this event, arisen from the longitudinally split short vertical bar of the cross, and the two large components from the split long vertical bar. The small components represent the *Y*-chromosome and the large ones the *X*-chromosome. Edward ('11) figures in *Ascaris felis* the *XY*-pair in the prophase quite similar to my figure 106 *B*. As stated previously, the *X*- and *Y*-chromosomes are separate in the first division and in figure 106 *D* from a late prophase they are already somewhat separated. A preliminary separation of the members of the *XY*-pair therefore takes place first in the prophase in advance of the other chromosome pairs. This may be correlated with the fact stated previously that the *X*- and *Y*-chromosomes are frequently found in the second metaphase side by side instead of joined together to form the usual unequal dyad.

2. *Notonecta irrorata*. The history of the ring in this species is the same as that described for the *M*-chromosome of *N. insulata* (fig. 107 *A-D*). The second longitudinal split remains here also during condensation, and although not seen in lateral view (fig. 107 *C*) is frequently visible in polar view of the metaphase (fig. 107 *D*; see also fig. 16). The crosses are likewise similar to those of the other species (fig. 108 *A-H*).

3. *Notonecta undulata*. A detailed study of the prophase figures in this species has not been attempted. They are evidently very different from those of *N. irrorata* and *N. insulata* and their irregular shape renders them difficult to trace. Some of these in the diffuse stage are shown in figure 109 *A-F*, and after they have condensed in figure 110 *A-F*.

C. Discussion

The prolonged discussion that has followed Flemming's original discovery of the open ring type of bivalent chromosome is even now not terminated, and the same is true of the cross described

by Paulmier and other early observers of the insects. In some respects *Notonecta* is not well adapted for the elucidation of this problem, owing to the difficulties attending the study of the chromosomes during most of the growth period. On the other hand, this form offers certain advantages in the fact that the formation of the rings and crosses may be clearly followed during the pro-phases. The facts here seen seem to leave no doubt that the rings are formed in essentially the same way as in the *Amphibia* and *Tomopteris*, though their relation to the original spireme can not be traced.

Those observers (Grégoire, the Schreiners, and many others) who accept a side-by-side conjugation, or parasynapsis, regard the ring as originating by the opening out of the longitudinally split spireme. Those observers (Paulmier, McClung, et al.) who accept an end-to-end conjugation, or telosynapsis, regard the ring as originating by the bending together of the split spireme at the two extremities. In either case, the final result is the same as far as the real significance of the ring is concerned. The plane between the two half rings passes through the synaptic point and therefore, according to most observers, a division in this plane means a reduction division, the division in the plane of the ring dividing it into two whole rings is longitudinal and equational. According to some observers; e.g., Paulmier, Montgomery, Farmer and Moore, and also most of the adherents of parasynapsis, the first division is reductional. McClung and his students, however, believe that in most *Orthoptera* it is the second division that is reductional. Bonnevie holds that the ring divides in its own plane in both divisions and that therefore there is no reduction; the rings of *Enteroxenus*, however, have been differently interpreted by the Schreiners ('07).

In *Notonecta* it is impossible to trace the chromosomes through the greater part of the growth period when they are aggregated in a karyosphere, but the evidence seems in favor of the hetero-homeotypic scheme of Grégoire. The ring is formed from two parallel rods which probably represent univalent chromosomes. The first division separates the ring into two half rings, and is therefore probably a reduction division. The second division is in

the plane of the ring and is therefore probably an equational division.

In many cases the ring goes on to the metaphase spindle without further modification, e.g., in the vertebrates and higher plants, annelids, etc., but in some cases, e.g., the insects and copepods, it condenses into a tetrad, as first explained in detail by Paulmier ('98). In other forms where the ring condenses, the split is either entirely lost before the second division or is only faintly indicated by an indentation. In most forms in which no condensation takes place, the identity of the chromosomes is lost in the interkinesis, although in *Tomopteris*, the Schreiners ('06a) have traced the second longitudinal split with some degree of certainty to the second division. In *Notonecta*, the ring condenses to form a tetrad, but the longitudinal split remains most distinct. In *N. insulata* in the case of two chromosomes and in *N. irrorata* in the case of one chromosome, in the first metaphase, the chromosome is completely divided into two parts and it remains thus until drawn on the second spindle. The second division follows directly on the first, the telophase of the first being the prophase of the second. Since this split can be traced from the early prophase of the first division to the second metaphase when it lies in the equatorial plane, there can be absolutely no question as to its identity with the division line of the second division.

The cross is in principle the same as the ring, as first pointed out by Paulmier ('98) and as more recently discussed from the point of view of parasynapsis by the Schreiners ('06 a,b) and Montgomery ('11), the difference in form being due to the divergence of the two parallel rods from the ends (cross) instead of in the middle (ring). In the process of condensation the cross becomes a typical tetrad in contrast to the ring-tetrad, whose quadripartite nature is not detectable in lateral view since the second longitudinal split lies in the plane of the spindle. The similarity between the two is easily seen however, if we compare a lateral view of the cross-tetrad with an end view of the ring-tetrad (cf. fig. 104 *H* with 95 *B*). The first division plane passes across the short axis of the cross-tetrad, and if we consider each original parallel rod as a univalent chromosome this is a reduc-

tion division for this figure as well as for the ring-tetrad. The second division plane coincides with the second longitudinal split and probably means an equational division. In the case of the parallel rods, similarly, the first division separates the two original components. The evidence therefore, is in favor of the first division acting reductionally for all the autosomes. This is not true, however, for the XY-pair, the two components of which are finally separated in the second division. The fact that the reduction and equational divisions are reversed in the case of the XY-pair and of the X-chromosome has been noted in many other cases. With this exception and with the exception of the multiple element of *Mermeria* (McClung '05), and possibly a few others (Blackman '10), all the chromosomes are believed to undergo a qualitative division at the same time.

There is some evidence from *N. insulata* for Baumgartner's ('04) view that the form of individual chromosomes in the prophase is constant. The two largest chromosomes assume a ring shape, several of the large ones become crosses, one of the large ones a double rod, and a small one a ring; the smallest ones could not be traced. The Schreiners ('06) have also concluded that to a certain point the form of a particular chromosome is constant. Davis ('08) in the Orthoptera and Blackman ('10) in the myriapods, hold the same view. On the other hand, Bonnevie ('07) in *Nereis* and Foot and Strobell ('05) in *Allolobophora*, believe that the form of the chromosome is merely a matter of chance. Bonnevie states that rings are limited to chromosomes of a certain size, and Robertson ('08) has attempted to show in *Syrbula* that shape is dependent on size. From the fact that both very large and small rings occur in *N. insulata*, it seems that in this case, form is not dependent on size.

VI. MITOCHONDRIA

A. Observations

1. *Late growth and division stages.* An exhaustive study of this subject has not been undertaken, but a brief treatment is given because of a few observations that I have to offer. Owing to the

precarious nature of the mitochondrial stain of Benda, only a few clearly differentiated slides were obtained. The general tone of both cytoplasm and karyoplasm is a pale lavender or rusty red, the chromatin is a brick red and the mitochondria a deep purple. In the drawings, the lavender is represented as a pale grey, the brick red as a darker grey, and the purple mitochondria as black and dark grey. The earliest stage of *N. insulata* obtained is shown in figure 111; the chromatin is in compact masses in the karyosphere, and the mitochondria are scattered through the cytoplasm. A mass of mitochondria from which project fibers, is attached to the nuclear wall. A slightly later stage is shown in figure 112, where the karyosphere is breaking up, and the nuclear plate of mitochondria has disappeared. From these figures, it is evident that the mitochondria are of two distinct kinds, fibers and spheres. The spheres occur chiefly around the nuclear periphery, and frequently form a complete circle about it. The fibers usually occur further out in the cytoplasm and tend to aggregate in several dense clusters. The relation between the fibers and the spheres is shown in figure 113; the spheres have a curved rod at the periphery extending about half way around the circumference, the rest of the sphere is less deeply staining. By a gradual disappearance of this less dense substance, the sphere is converted into a fiber, or rather, the fiber which was already in the sphere becomes free. Whether the fibers always originate in this way, it is impossible to say. In figure 114 is represented a metaphase of the first division in side view. As the asters form, the mitochondria become pushed away from their vicinity although a few of the fibers take up a position along the astral rays. In the division stages, the mitochondria are quite evenly distributed through the cytoplasm between the mitotic figure and the cell wall, though there is usually a clear area at the periphery of the cell. The spheres and fibers are more intermingled than during the growth stages. When the cell divides, the mitochondria are divided en masse, so that each daughter cell receives approximately the same amount (fig. 115). There is no evidence that individual fibers or spheres divide, except possibly in the region of constriction. In the interkinesis the mitochondria

are distributed through the cytoplasm, so that in the second division they are arranged as in the first. They are divided again en masse when the cell divides.

2. *Early growth stages: nuclear plate.* In all three species of *Noto-necta*, there is present during the greater part of the growth period, a characteristic deeply staining mass applied to the nuclear wall. This takes the chromatin stains of haematoxylin and safranin, but is purple when stained according to Benda's method, and is evidently of mitochondrial nature. In the earliest growth stages, the body is more or less spherical, and may be closely applied to the nuclear wall, or may lie free in the cytoplasm (figs. 43, 44, 63-66). The mitochondrial body flattens down so as to form a plate on the outside of the nuclear membrane; it is in this form during the spireme stage (figs. 45-53, 67, 74). In *N. undulata*, at the time when the spireme is disappearing, there is a peculiar bulging of the nuclear membrane at the place where the nuclear plate is attached (fig. 68). On the nuclear side, chromatic substance is present in the swelling, and in the cytoplasm there is a mass of mitochondria in this region; this differentiation is clear with the Benda stain (fig. 116). Up to this time, there are practically no mitochondrial bodies present except the nuclear plate. The mitochondrial mass which appears outside the nuclear plate is composed of small spheres and fibers. The bulging very soon disappears, the plate flattens down again with the membrane (fig. 69), and the mitochondria become distributed through the cytoplasm (fig. 117). The nuclear plate gradually disappears; in *N. insulata* it becomes conical or spherical in the later stages and apparently may separate from the nuclear membrane (fig. 55).

In haematoxylin preparations, the plate when viewed from above appears as a spongy mass (fig. 53 *B*). When viewed from the side one or two small granules in many cases are seen projecting from the surface; these may be centrosomes. This is suggested further by the peculiar modification of the protoplasm in their vicinity, giving the appearance of an idiozome or attraction-sphere which lies as a cap over the nuclear plate. The origin of these granules cannot be conclusively shown, although in the early stages a small granule may be often detected in the modified

protoplasm near the mitochondrial body. It seems altogether probable that the centrosome becomes embedded in the mitochondrial mass at an early stage and remains in connection with it during the growth period. It is of interest to note that the orientation of the spireme is not toward the nuclear plate but toward the karyosphere; these two bodies may lie in any position relative to each other, the karyosphere being usually eccentrically placed.

B. Discussion

Mitochondria have been found in many invertebrates and vertebrates in both germ and tissue cells by many observers; Fauré-Frémiet ('10), Prenant ('10) and Montgomery ('11) have recently given comprehensive reviews of the subject so that only a few points will be touched on here. Most commonly, mitochondria appear as fine granules which have a tendency to arrange themselves in rods or rhondromites (Meves '00). In some forms, the mitochondria form long fibers called by Meves '08) 'chondriokonts.' In a few cases the mitochondria have been described as vesicles with a dense shell, e.g., by Meves in *Pygaera* '00) and other forms, by Meves and Duesberg ('08) in *the hornet*, and by Gérard ('09) in *Stenobothrus*. In the latter case the mitochondria occur both in the form of vesicles and fibers and bear a striking resemblance during the growth period to those of *Notonecta*. In *Notonecta* it is perfectly clear that the fibers are formed not by chains of granules but directly from the vesicles by a disappearance of the surrounding substance; the dense shell described by the above named observers is probably the mitochondrial fiber in the sphere. Loyez ('09) has found in the egg of tunicates that the mitochondrial fiber develops into a yolk sphere; this is practically the reverse of what occurs in *Notonecta*.

In division the mitochondria usually appear to be divided en masse, as they are in *Notonecta*; but in some of the Protozoa, according to Fauré-Frémiet ('10), the individual mitochondria divide at the time of the division of the micronucleus. In some of the Metazoa they form a mantle of long fibers at the side of the spindle and are divided individually and equally. According

to the observations of Benda ('02) and others, no mitochondria occur in the spindle itself. In *Notonecta* this is true to a certain extent, but a few mitochondrial fibers lie along the inside spindle fibers.

In regard to the source of the mitochondria, the evidence from *Notonecta* leads to the conclusion that the first mitochondrial body is of cytoplasmic origin, that this becomes applied to the nuclear wall, and that by an interaction of this material and some of the chromatic material of the nucleus, the numerous mitochondrial bodies of the later growth period are formed. The mitochondria are not of nuclear origin in the sense of Hertwig for the chromatin in the nucleus and the mitochondria outside are of very different appearance. But it seems quite probable that their chief elaboration takes place under the influence of the chromatin since this accumulates in the region where they are formed and at the time of their formation. It seems to me probable that the mitochondria are merely early formed cytoplasmic structures which function in the mature sperm.

The observations on the nuclear plate in the American species confirm in the main the observations of Pantel and Sinéty on *N. glauca*. The 'archoplasmic vesicles' which they find scattered through the cytoplasm are no doubt the mitochondrial spheres described above; these form part of the acrosome of the sperm. The granular masses, 'matériel nebenkernien simple' which forms the principal foundation of the nebenkern, are probably identical with the masses of mitochondrial filaments which I have described; the fibrous nature of the masses is brought out by the Benda stain.

VII. SUMMARY

1. The most suggestive result of the foregoing observations is to show that in the case of *Notonecta* the change in the number of the chromosomes from species to species can be explained by the relations of two particular chromosomes. In *N. undulata* these two chromosomes are always separate, in *N. irrorata* always united to form a single body, while in *N. insulata* they may be separate in the first spermatocyte division, but are united in the second.

2. In all three species all the chromosomes are aggregated during the growth period to form a massive karyosphere, which consists of chromatic bodies embedded in plasmasome material. The precise origin of this body is somewhat difficult to ascertain, but the evidence indicates that it contains at least part of the early spireme.

3. In the prophases the chromosomes are formed from the karyosphere, which gives rise to dense chromatic bodies, which form diffuse double threads; these condense to form ring- and cross-tetrads, etc., whose entire history can in some cases be traced.

4. Mitochondria are present in the form of a flat plate in the early stages, and of spheres and fibers later; the fibers may arise directly from the spheres. The mitochondria are divided en masse with cell division.

September, 1912

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⁵ In order to abbreviate the literature list, many papers have been referred to in general, and are not included in the list.

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PLATE 1⁶

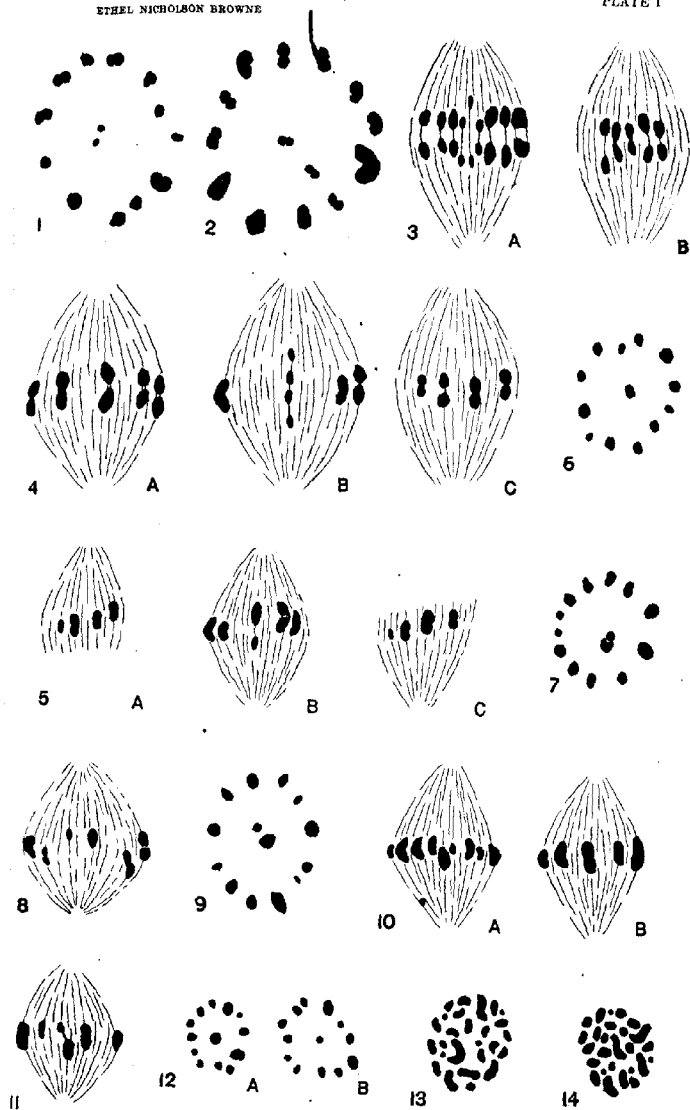
EXPLANATION OF FIGURES

Notonecta undulata

× 2250

- 1-2 Metaphase of first division, polar view, showing 14 chromosomes, two small ones in center.
- 3 A, B Serial sections of spindle in side view, early anaphase, first division.
- 4 A, B, C Same, two central pairs arranged linearly.
- 5 A, B, C Serial sections of spindle in side view, initial anaphase, second division, showing 13 chromosomes, XY in center.
- 6-7 Metaphase of second division, polar view.
- 8-9 X and Y on separate fibers.
- 10 A, B Same in complete spindle.
- 11 X and Y connected by oblique fiber.
- 12 A, B Sister anaphase groups of second division, from same spindle.
- 13-14 Spermatogonial groups showing 26 chromosomes.

⁶ All the figures (except fig. 113) were drawn with the camera lucida. In some cases, for the sake of clearness, overlying chromosomes have been displaced.



Browne, del.

PLATE 2

EXPLANATION OF FIGURES

Notonecta irrorata

× 2250

- 15-16 Metaphase of first division, polar view, showing 13 chromosomes, one small one in center.
- 17 *A, B, C* Serial sections of spindle in side view, early anaphase, first division.
- 18 Components of central pair on different fibers.
- 19 *A, B, C* Serial sections of spindle in side view, initial anaphase, second division, showing 12 chromosomes, *XY* in center.
- 20 Metaphase of second division, polar view.
- 21 *A, B, C* Complete spindle, *X* and *Y* on different fibers.
- 22 Same, polar view.
- 23 *A, B* Sister anaphase groups of second division, from same spindle.
- 24 Spermatogonial group, showing 24 chromosomes.

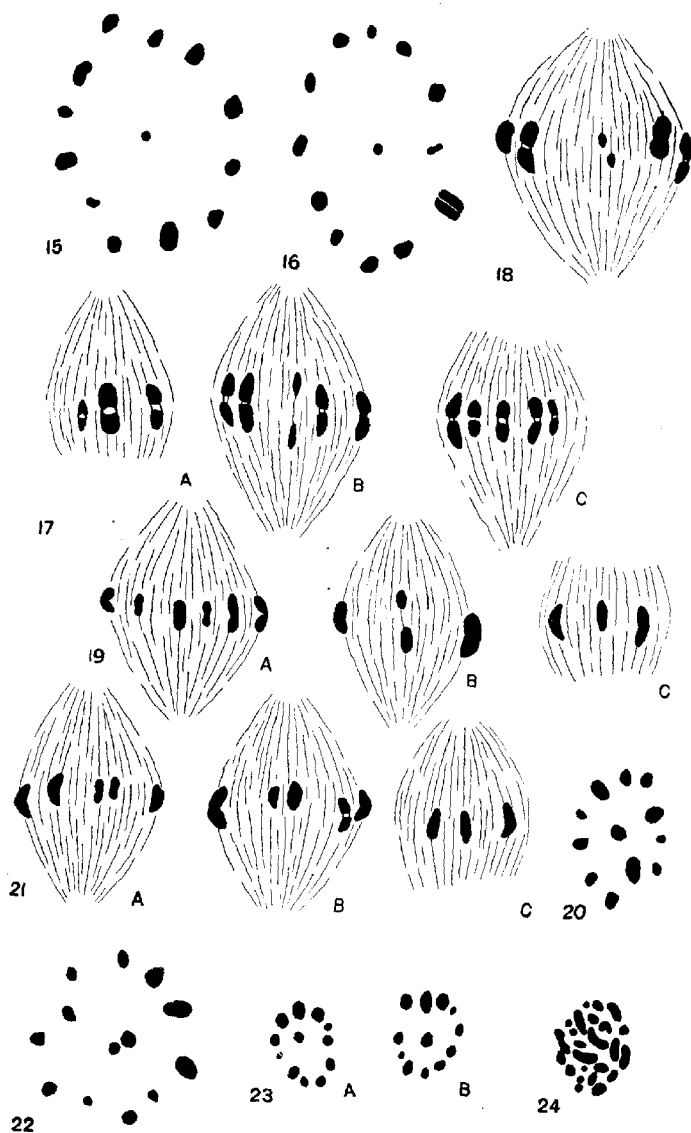


PLATE 3

EXPLANATION OF FIGURES

Notonecta insulata

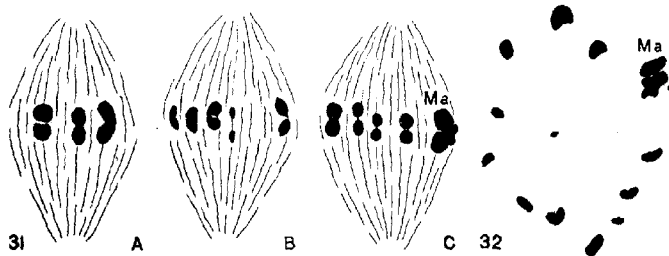
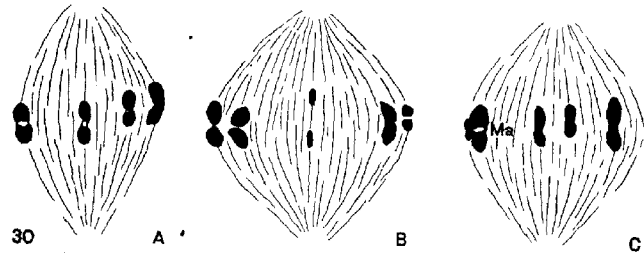
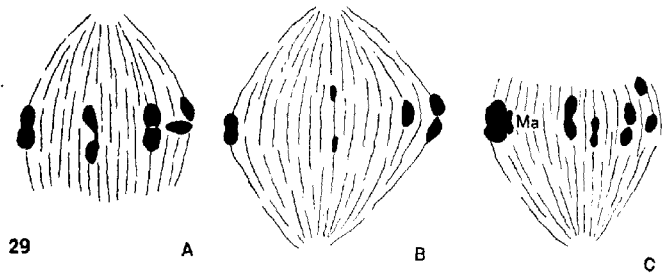
× 2250

25-26 Metaphase of first division, polar view, showing 14 chromosomes, two small ones in center.

27-28 Same with 13 chromosomes, one small one in center.

29-31 *A, B, C* Serial sections of entire spindles in side view, showing one small pair in center, and compound chromosome Ma, consisting of largest chromosome and second small one.

32 Polar view, showing same.



Browne, del.

PLATE 4

EXPLANATION OF FIGURES

Notonecta insulata

× 2250

33 *A, B, C* Serial sections of entire spindle in side view, showing two small pairs in center, components of *Ma* separate.

34 *A, B, C* Same, two small pairs arranged linearly.

35-36 Metaphase of second division, polar view, showing 12 chromosomes, *XY* in center.

37 *A, B* Complete spindle, initial anaphase, side view.

38-39 *X* and *Y* on separate fibers.

40 *A, B* Sister anaphase groups of second division, from same spindle.

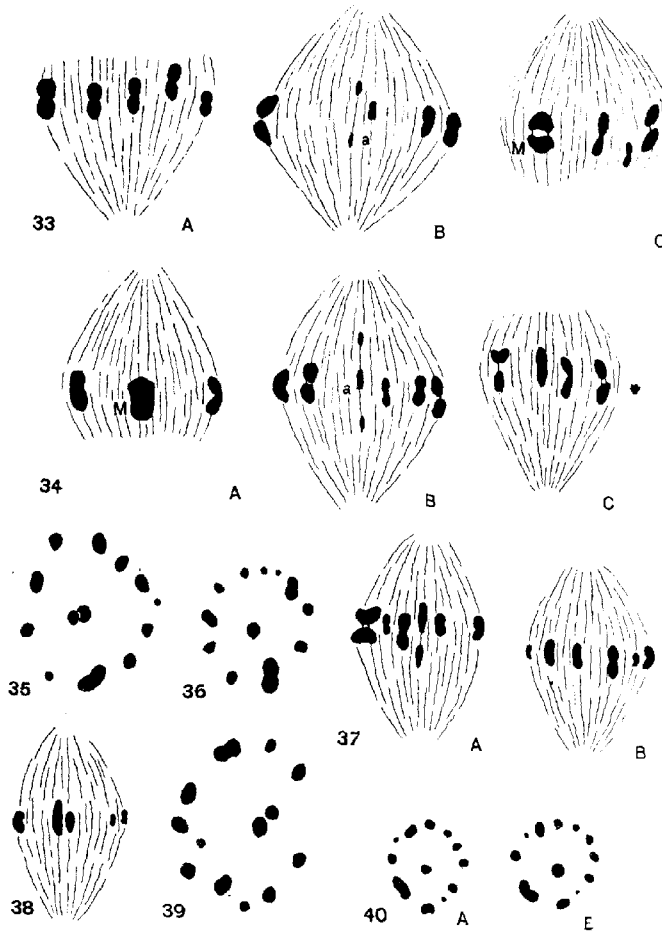


PLATE 5

EXPLANATION OF FIGURES

Growth stages. *N. insulata*

- 41 Very young spermatocyte. $\times 2250$.
- 42-43 Formation of scattered masses of chromatin; mitochondrial body lies outside nuclear membrane in fig. 43. $\times 2250$.
- 44-45 Formation of spireme; mitochondrial plate in fig. 45. $\times 2250$.
- 46 Oriented spireme. $\times 2250$.
- 47 Withdrawal of spireme loops from karyosphere. $\times 2250$.
- 48-50 Disappearance of spireme loops. $\times 2250$.
- 51 Same as fig. 41, drawn to scale of figs. 52-53, to show increase in size of nucleus during growth. $\times 1350$.
- 52-56 Structure of karyosphere during late growth period. $\times 1350$.
- 53 *B* Nuclear plate viewed from above. $\times 2250$.
- 56 From living material. $\times 1350$.
- 57 Irregular karyosphere before formation of aster; mitochondrial body has disappeared. $\times 1350$.
- 58 Karyosphere rounded, after formation of aster. $\times 1350$.
- 59 Chromatin leaving plasmasome. $\times 1350$.

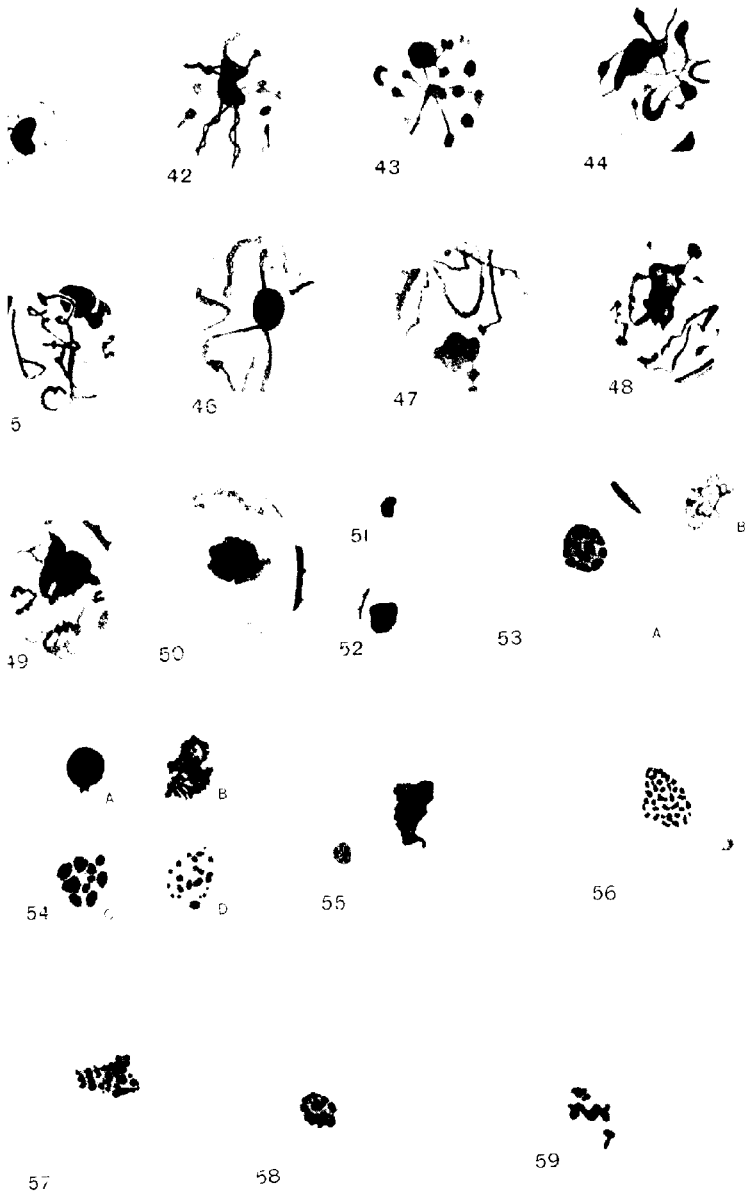


PLATE 6

EXPLANATION OF FIGURES

Dissolution of karyosphere, *N. insulata*.

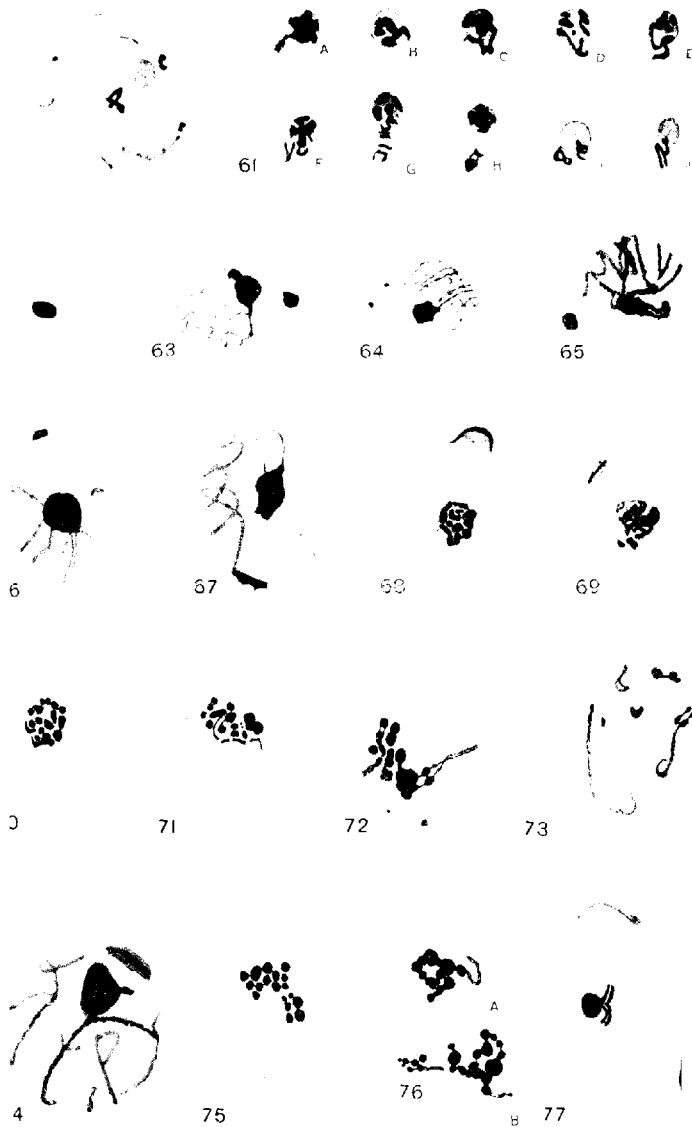
60. Last chromatin mass. *H*-chromosome leaving plasmasome, diffuse chromosomes at periphery. $\times 1350$.
 61. *A*. Chromatin leaving plasmasome. $\times 1350$.

Growth stages, *N. undulata*.

62. Very young spermatocyte. $\times 2250$.
 63. Leptotene-spireme; mitochondrial body outside nucleus. $\times 2250$.
 64. Synthesis stage. $\times 2250$.
 65-66. Oriented spireme. $\times 2250$.
 67. Withdrawal of spireme loops from karyosphere; mitochondrial plate. $\times 2250$.
 68. Karyosphere fully formed; protrusion from nuclear wall in region of mitochondrial plate. $\times 1350$.
 69-70. Karyosphere breaking up into balls. $\times 1350$.
 71-73. Thread formation. $\times 1350$.

Growth stages, *N. inornata*.

74. Looped spireme. $\times 2250$.
 75. Karyosphere broken up into balls. $\times 1350$.
 76-77-77. Thread formation; deeply staining plasmasome in Fig. 77. $\times 1350$.



Reprints 50c.

PLATE 7

EXPLANATION OF FIGURES

× 2250

Prophases. *N. insulata*

- 78 Early prophase, chromosomes diffuse on nuclear wall, *M* condensed.
- 79 Later prophase, rings and crosses.
- 80 Late prophase, chromosomes fully condensed.
- 81 Just before metaphase.

N. irrorata

- 82 Early prophase, diffuse stage.
- 83 Later prophase, rings and crosses.
- 84 Very late prophase, spindle forming.

N. undulata

- 85 Early prophase, diffuse stage.
- 86-87 Condensation, irregular figures.
- 88 Very late prophase.

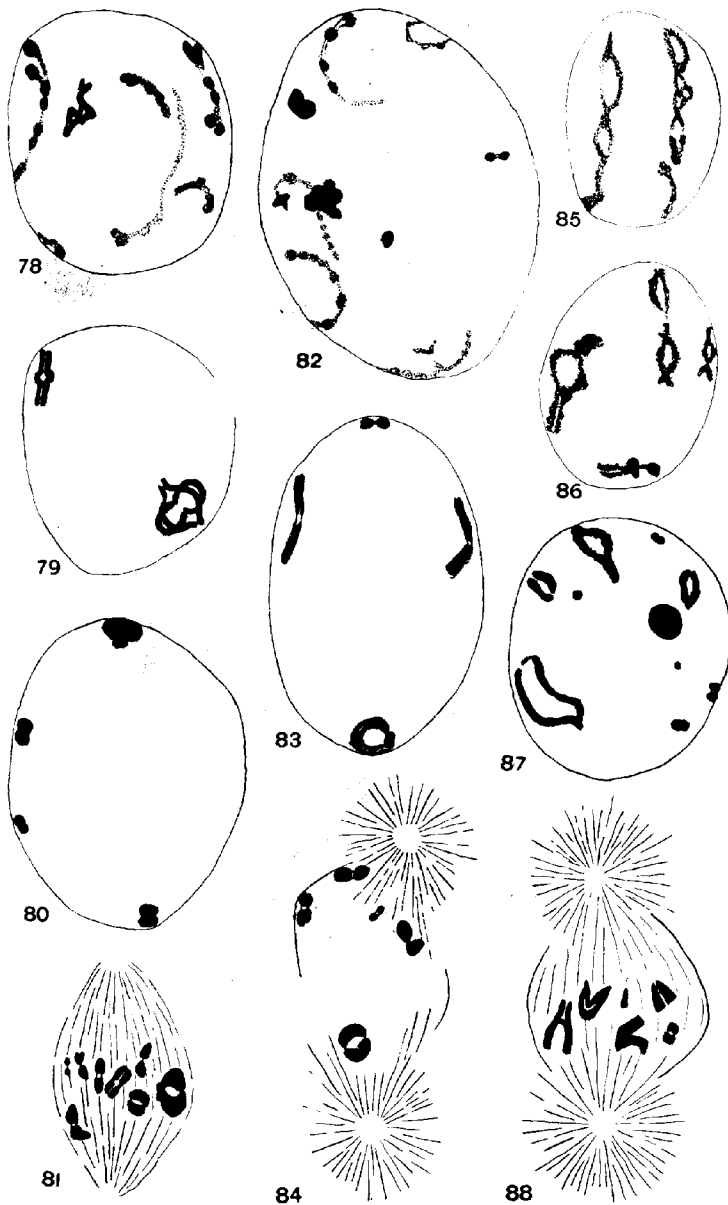


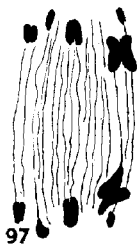
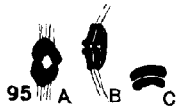
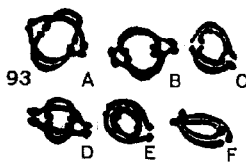
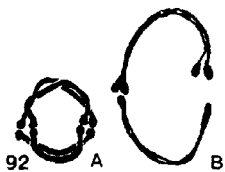
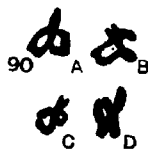
PLATE 8

EXPLANATION OF FIGURES

× 2250

Ring tetrad. *N. insulata*

- 89 *A-D* *M*-chromosome as it leaves plasmosome.
90 *A-D* Opening out of two bars from middle.
91 *A-D* Small ring formed; in *B*, second split has come in.
92 *A, B* Open double ring.
93 *A-F* Ring condensing.
94 *A-D* Condensed ring of late prophase.
95 Ring tetrad in metaphase; *A*, side view; *B*, end view; *C*, polar view.
96 Ring tetrad in initial anaphase; *A*, slightly oblique view; *B*, end view, showing compound nature of chromosome.
97 Late anaphase, each part of *M* split.
98 *M* as it is drawn on second spindle.
99 Initial anaphase of second division, showing *M* dividing along split.
100 Late anaphase.
101 *A-F* Second largest chromosome forming ring tetrad; *D*, late prophase; *E*, metaphase, side view; *F*, polar view.
102 Telophase of first division, showing two largest chromosomes longitudinally split.



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PLATE 9

EXPLANATION OF FIGURES

× 2250

Tetrad formation. *N. insulata* (continued)

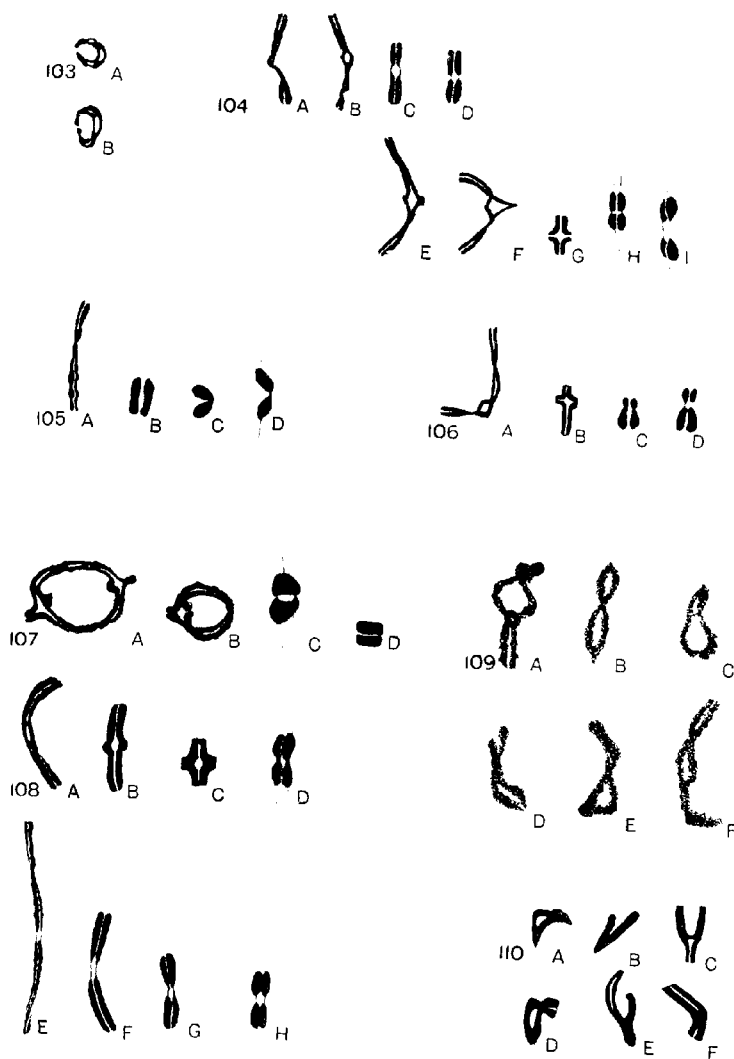
- 103 *A, B* Small ring.
104 *A-I* Formation of cross tetrad; *H*, in metaphase; *I*, in anaphase.
105 *A-D* Condensation of double rod.
106 *A-D*, Condensation of *XY*; *D*, late prophase, elements separating.

Tetrad formation. *N. irrorata*

- 107 *A-D* Condensation of ring; *C*, in metaphase, side view; *D*, polar view.
108 *A-H* Formation of cross tetrad; *D*, in metaphase.

Prophase figures. *N. undulata*

- 109 *A-F* Diffuse stage.
110 *A-F* Condensed stage.



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PLATE 10

EXPLANATION OF FIGURES

× 1350

Mitochondria. Chromatin represented by dark grey, mitochondria by black and grey. *N. insulata*

- 111 Late growth, mitochondrial mass attached to nuclear wall.
- 112 Later stage.
- 113 Transition stages between spheres and fibers. Free hand drawing.
- 114 Metaphase, first division.
- 115 Division of mitochondria en masse with cell division.

N. undulata

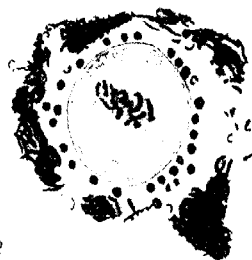
- 116 Early growth, formation of mitochondria near nuclear bulge.
- 117 Mitochondria becoming evenly distributed.

MALE GERM CELLS IN NOTONECTA
ETHEL NICHOLSON BROWNE

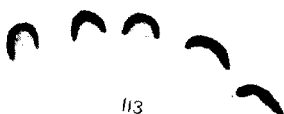
PLATE 10



111



112



113



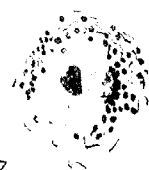
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115



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STUDIES ON THE EFFECTS OF ALCOHOL, NICOTINE AND CAFFEINE ON WHITE MICE

II. EFFECTS ON ACTIVITY

L. B. NICE

From the Laboratory of Physiology in the Harvard Medical School

THREE FIGURES

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HISTORICAL

During the past twenty years the effects of drugs on the activity of men and animals have been studied by many investigators. With but few exceptions however, these studies have extended over short periods of time and the drugs have been given intermittently.

Alcohol

Hodge ('03) compared the spontaneous exercise of a pair of alcoholic and a pair of control dogs and found that the alcoholic female exercised 57 per cent as much as the control female and the alcoholic male 71 per cent as much as the control male. Their activity was measured by means of pedometers in their collars.

Stewart ('98) gave two grey rats 20 per cent alcohol to drink. The activity of the alcoholic rats, as measured by revolving cages, surpassed that of the controls. Thirty per cent alcohol decreased the activity of white rats.

In investigations on men Lombard ('92), Frey ('96), Kraepelin ('99), Rossi ('94) and Schumburg ('99) showed that small doses of alcohol increased the amount of work done with the ergograph. Schnyder ('03) observed that alcohol when taken in a fasting condition, increased the amount of work done with the ergograph but when taken after or during a meal decreased it. With Hellsten ('04) 80 grams of absolute alcohol diminished the amount of work he could perform. These doses were so large, however, that they produced disturbances of digestion. Aschaffenburg ('96) found that wine decreased the efficiency of typesetters. Rivers ('08) considers that the increase of work noted by the above investigators under the influence of alcohol was due to faulty methods. The interest of taking the alcohol stimulated the subjects to extra exertions. His own experiments were carried on with control mixtures so the subjects did not know when they were taking alcohol. He states that "small doses, varying from 5 to 20 cc. of absolute alcohol have no effect on the amount or nature of the work performed with the ergograph, either immediately or within several hours of their administration."

Nicotine

So far as I can find, no experiments have been made on testing the effects of nicotine on muscular activity. Tobacco was found by Lombard ('92), Harley ('94), Féré ('04) and Rivers ('08) to decrease the amount of muscular work as recorded by the ergograph, although the pleasurable sensations connected with smoking would be expected to stimulate the subject and thus increase the amount of work done.

The fact that tobacco is forbidden to athletes when in training for tests that require great muscular strength shows that it is generally considered to have a depressing effect on muscular activity.

Caffeine

Caffeine was shown by Mosso ('93), Koch ('94), Hoch and Kraepelin ('96), Schumburg ('99) and Rivers ('08) to increase the capacity for work with the ergograph. From the results of these experiments Rivers ('08) says:

This stimulating action persists for a considerable time after the substance has been taken without there being any evidence, with moderate doses, of reaction leading to a diminished capacity for work, the substance thus really diminishing and not merely obscuring the effects of fatigue. When taken in excess the stimulating action may be so transitory, and followed by so great a decrease that it may legitimately be spoken of as an accelerator of fatigue.

METHODS

This study was undertaken to find the effects of alcohol, nicotine and caffeine on the spontaneous activity of white mice when kept under the influence of these drugs in moderate quantities all the time.

Sixteen male mice eight weeks old were used in the experiment. They were all descendants of one pair of mice whose offspring had been inbred for four generations. These mice belonged to the fourth generation and came from four different lots, each lot being the young of one male and several females. One mouse from each lot was placed in each of the experimental lines. Thus mice of the same sex, the same age, and very closely related were the subjects of this investigation.

Four lines were carried: one was given alcohol, a second nicotine, a third caffeine and a fourth was carried for controls. The alcohol and nicotine were given in the same proportions as in preceding experiments, as on these strengths the mice seemed to keep in good health. The caffeine was given in a 1:300 solution.

Each mouse in the alcohol line was given 35 per cent alcohol to drink instead of water, and every other day, 3 cc. of 35 per cent alcohol was added to its crackers and milk.

Each mouse in the nicotine line received 1:1000 nicotine sulphate solution to drink instead of water, and had 3 cc. of 1:1000 nicotine sulphate added to its crackers and milk every other day.

In the caffeine line each mouse drank 1:300 caffeine citrate solution instead of water, and every other day 3 cc. of 1:300 caffeine solution was added to the crackers and milk.

All of the sixteen mice were given the same food which consisted of buckwheat and oats, every other day crackers and milk, and once or twice a week meat.

The experiment continued from November 18 to June 8. During the winter months the room was heated with hot water and remained at about 65° F.

To study the spontaneous activity of these mice revolving cages were devised. These cages are similar to those used by Stewart ('98) and later by Slonaker ('07, '12) in their studies on rats. The cages are 6 inches wide by 10 inches in diameter, and made of 8-mesh galvanized wire. Each cage is fastened to an axle which revolves with the cage. The axle is $\frac{1}{4}$ inch in diameter and 18 inches long. The ends of the axle are pointed and set into the end of a bored out set screw forming a pinion which by reducing friction permits the cages to revolve very easily. By means of turning the set screw the pinions can be adjusted in case of wear. The cages are mounted as shown in the accompanying photograph (fig. 1).

The revolutions of each cage are recorded by means of an alarm clock whose balance wheel had been removed. A wire about 6 inches long is attached to the escapement lever of the clock and to one end of a wooden lever which rests on the axle near one end of a cage. In one brass hub of each cage two pins are set on opposite sides of the axle, and $1\frac{1}{2}$ inches from it. These pins are parallel with the axle. As a cage revolves the end of the wooden lever is raised by each pin in turn causing the clock to register. Each revolution of a cage corresponds to one second on the clock.

Each cage is supplied with a nest box made of galvanized tin $2\frac{3}{4}$ inches wide, $2\frac{3}{4}$ inches long and 2 inches deep. These are swung to the axle by two wire hooks attached to the top of the nest box near its ends. A tunnel $1\frac{1}{2}$ inches square having a wire mesh floor leads to the opening in one end of the nest box where the mouse enters. The floor of the nest box is a hinged door.

On the top of the nest box is a feed box 3 inches long and 1 inch wide with two compartments, one for grain, the other for crackers and milk. The feed box is held in place by a spring clip. Another clip holds a small wide mouthed bottle which is inverted. This bottle contains the water or drug which the mouse drinks. In the mouth of this bottle is a rubber stopper with an opening through it $\frac{1}{2}$ inch in diameter. Through this opening a glass tube

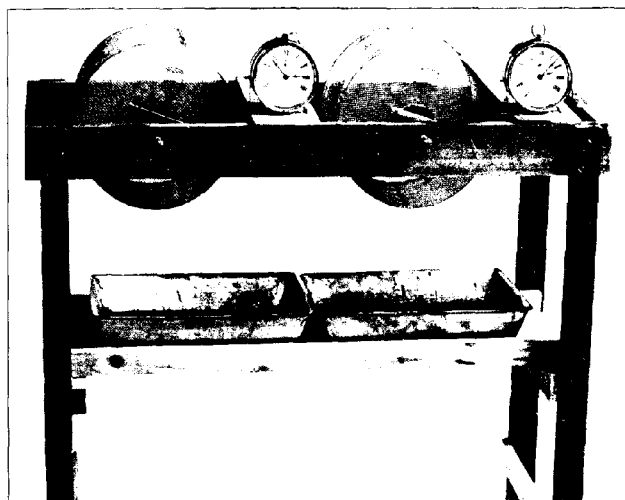


Fig. 1 Revolving cages and recording clocks

$\frac{3}{4}$ inch in diameter inside is inserted. The lower end of the glass tube is drawn towards a point making an opening $\frac{1}{4}$ inch in diameter. This device compelled all the mice to drink directly from the bottles.

THE GROWTH OF THE MICE

The mice were weighed at the beginning of the experiment when they were eight weeks old. They were weighed once a week during the next two months and once each month the next five months.

TABLE 1
Weight of the mice in grams

Dose	No.	Weight at various intervals									
		0 weeks	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks
Control	1	19.0	20.0	20.0	20.0	21.5	21.0	22.5	21.5	25.0	25.0
	2	19.5	18.0	20.0	20.0	22.5	23.5	23.5	25.0	25.0	25.0
	3	20.0	19.0	22.0	22.0	25.0	25.0	28.0	32.0	33.0	35.0
Alcohol	4	19.0	17.0	18.5	19.0	21.5	22.0	22.5	21.5	21.5	21.0
	5	16.5	20.0	19.0	19.0	20.5	21.5	21.0	19.0		
	6	18.5	18.0	18.0	19.0	21.5	23.5	25.0	25.5	25.0	
Nicotine	7	18.0	19.0	19.5	19.0	20.5	22.0	23.5	21.5	27.0	19.0
	8	18.0	19.0	19.0	19.0	21.0	22.5	21.5	21.5	25.0	20.0
	9	16.5	20.0	19.5	21.0	21.5	22.0	21.5	21.5	21.0	23.0
Caffeine	10	18.0	15.0	17.0	18.0	18.5	19.0	19.0	20.0	22.0	20.0
	11	19.0	17.0	17.0	17.5	18.0	21.0	22.0	21.5	25.5	23.0
	12	20.0	17.0	17.5	18.0	19.0	20.5	20.0	21.5	22.0	21.5
Coffeine	13	16.5	17.0	20.0	20.0	19.0	22.5	21.0	25.0	26.5	27.0
	14	17.0	17.0	18.5	18.0	19.0	20.5	20.5	19.5	17.0 ¹	
	15	16.0	16.0	17.0	18.0	19.0	22.5	23.0	23.5	26.0	26.0
	16	15.5	15.0	19.0	19.5	20.0	21.5	21.0	21.5	26.0	30.0

¹ Mouse No. 5 died at the end of the twenty-seventh week, No. 6 at the end of the thirty-fourth week and No. 14 in the thirty-second week.

At eight weeks the control mice averaged 19.5 grams in weight, the alcohol mice 18 grams, the nicotine mice 18.5 grams and the caffeine mice 16 grams. At twenty-four weeks the control mice averaged 24 grams, the alcohol mice 24 grams, the nicotine mice 22 grams and the caffeine mice 23 grams. At thirty-six weeks the control mice averaged 27.3 grams, the alcohol mice 19.5 grams, the nicotine mice 22 grams and the caffeine mice 27.6 grams.

The control mice gained 4.5 grams each in the first sixteen weeks of the experiment and 3.3 grams in the last twelve weeks. The alcohol mice had gained 6 grams each when twenty-four weeks old, but had lost 4.5 grams on an average at the end of the experiment. The nicotine mice gained 3.5 grams each by the twenty-fourth week, and had gained no more at the end of the experiment. The caffeine mice gained the most of all, 7 grams in the first sixteen weeks and 4.6 grams in the last twelve weeks.

The average gain for the alcohol mice was 1.5 grams, for the nicotine mice 3.5 grams, for the control mice 7.8 grams and for the caffeine mice 11.6 grams.

WEEKLY ACTIVITY OF THE MICE

The number of revolutions registered by each clock was recorded at 12.30 P.M. every day. In the following tables the number of revolutions that each mouse ran are recorded by weeks.

Mouse No. 1 exhibited its greatest activity from the tenth to the eighteenth week, then became somewhat less active, increased again at the twenty-fifth week and then decreased rather gradually till the end of the experiment. Its maximum run was 139 078 revolutions at the thirteenth week and its minimum run was 48 380 revolutions at the thirty-fourth week. Its average run per week was 92 004 revolutions.

No. 2 exhibited its greatest activity from the eleventh to the seventeenth week and then had a second period of high activity from the twenty-fifth to the twenty-ninth week. Its maximum run was 147 576 revolutions at the fourteenth week and its minimum run was 59 320 revolutions at the thirty-fifth week. Its average run per week was 95 622 revolutions.

TABLE 2
Number of revolutions per week of the mice in the control line

AGE OF MICE IN WEEKS	MOUSE NO. 1	MOUSE NO. 2	MOUSE NO. 3	MOUSE NO. 4
9	80 243	78 785	55 718	63 878
10	120 935	82 528	71 041	123 370
11	122 119	118 306	99 070	139 978
12	132 593	125 001	100 003	120 495
13	139 078	140 830	81 004	101 626
14	126 355	147 576	98 966	80 772
15	124 957	131 274	81 620	84 200
16	94 870	129 195	80 028	94 145
17	92 598	119 668	85 370	109 275
18	108 405	91 304	69 584	129 304
19	86 990	84 900	73 100	144 913
20	66 117	83 987	78 744	105 902
21	74 287	85 647	58 647	135,978
22	72 890	64 281	58 119	130 395
23	82 120	85 955	76 740	125 572
24	88 454	93 540	54 545	68 147
25	107 148	109 515	54 532	126 018
26	91 722	101 215	60 060	116 095
27	93 892	74 380	54 104	105 780
28	88 755	110 640	55 180	100 500
29	67 071	119 481	41 058	78 013
30	77 439	63 365	40 881	84 547
31	88 028	81 600	47 324	94 140
32	85 345	85 102	52 164	105 615
33	72 652	74 825	36 155	91 057
34	48 380	72 453	39 825	77 830
35	74 460	59 320	40 746	62 100
36	69 195	63 554	41 247	57 633
Average	92 001	95 622	63 770	102 045

No. 3 was the least active of all the controls. It reached its maximum of 100 003 at the twelfth week and decreased steadily to the end of the experiment. Its minimum run was 36 155 revolutions at the thirty-third week. Its average weekly run was 63 770 revolutions.

No. 4 was the most active of the control mice. It showed high activity from the tenth to the thirteenth week, from the seventh to the twenty-third week, from the twenty-fifth to the twenty-eighth week and finally at the thirty-second week. Its

maximum run was 144 913 revolutions at the nineteenth week and its minimum run was 57 633 revolutions at the thirty-sixth week. Its average run per week was 102 045 revolutions.

Mouse No. 5 ran its maximum amount of 125 301 revolutions during the eleventh week and decreased rather rapidly till the twenty-seventh week when it died. Its minimum run was 2464 revolutions the twenty-sixth week. Its average run per week was 57 615 revolutions.

TABLE 3
Number of revolutions per week of the mice in the alcohol line.

AGE OF MICE IN WEEKS	MOUSE NO. 5	MOUSE NO. 6	MOUSE NO. 7	MOUSE NO. 8
9	57 634	41 370	28 430	45 395
10	74 915	81 694	76 230	122 193
11	125 301	58 444	132 113	131 473
12	56 542	15 404	96 680	93 965
13	104 745	31 693	142 762	61 901
14	34 380	32 522	128 495	83 629
15	80 092	63 128	129 393	86 196
16	69 722	79 258	113,560	83 327
17	74 074	86 895	117 005	83 658
18	56 969	69 616	99 138	72 748
19	52 830	71 204	96 925	68 684
20	57 087	57 318	112 247	81 660
21	51 785	54 427	97 093	61 738
22	47 297	63 423	89 195	69 655
23	45 628	66 502	96 835	77 245
24	43 680	71 510	76 733	67 364
25	56 831	75 558	97 210	67 607
26	2 464	54 630	85 420	66 063
27	2 700 ¹	63 116	67 990	29 535
28		69 428	67 600	27 432
29		51 997	51 083	12 728
30		46 795	49 736	177
31		70 095	71 074	25 638
32		69 675	74 660	50 189
33		33 820	54 982	29 610
34		21 315 ¹	60 107	3 610
35			58 600	5 012
36			62 650	13 001
Average.....	57 615	62 435	86 934	57 908

¹ Nos. 5 and 6 died at the end of the twenty-seventh and thirty-fourth week, respectively.

No. 6 ran uniformly low; its maximum run was 86 895 revolutions at the seventeenth week and its minimum run for an entire week was 15 404 revolutions the twelfth week. It died the thirty-fourth week. Its average weekly run was 62 435 revolutions.

No. 7 was the most active of the alcohol line. It showed great activity from the eleventh to the twentieth week. Its maximum was 142 762 revolutions at the thirteenth week and its minimum, not counting the first week, was 51 083 revolutions during the twenty-ninth week. Its average run per week was 86 934 revolutions.

No. 8 ran its maximum of 131 473 revolutions at the eleventh week. It decreased steadily and near the end of the experiment made some very small runs, of which 177 revolutions at the thirtieth week was the least. Its average weekly run was 57 908 revolutions.

No. 9 showed great activity from the tenth to the twentieth week, from the twenty-fourth to the twenty-seventh week and again the thirty-second week. Its maximum run was 134 027 revolutions at the eleventh week and its minimum run, not counting the first week was 67 089 revolutions at the thirty-fifth week. Its average weekly run was 99 693 revolutions.

No. 10 was the least active of this line; it rose gradually to its maximum run of 106 697 revolutions at the fourteenth week and then decreased to its minimum run of 10 253 revolutions at the twenty-ninth week, but increased somewhat later. Its average run per week was 58 843 revolutions.

No. 11 shows a reversed record, for it ran low until its twenty-seventh week. Its maximum run was 111 132 revolutions at the thirty-fifth week and its minimum run 14 947 at the thirteenth week when many of the mice were running their highest. Its average weekly run was 65 006 revolutions.

No. 12 exercised the most of all the sixteen mice. It reached its maximum of 192 395 revolutions at the thirteenth week and kept up its great activity throughout the experiment, not going below 100 000 revolutions until the thirty-fourth week. Its minimum run was 54 392 revolutions at the twelfth week. Its average run per week was 124 886 revolutions.

TABLE 4

Number of revolutions per week of the mice in the nicotine line

AGE OF MICE IN WEEKS	MOUSE No. 9	MOUSE No. 10	MOUSE No. 11	MOUSE No. 12
9	25 227	36 370	44 240	75 805
10	112 286	68 748	31 218	69 467
11	134 027	53 430	37 226	122 325
12	127 474	75 451	19 167	154 392
13	107 162	98 414	14 947	192 395
14	119 168	106 697	40 222	175 052
15	122 093	94 483	33 564	145 488
16	133 615	93 275	29 488	160 474
17	122 570	80 278	61 232	135 952
18	123 667	77 205	69 841	150 792
19	110 990	82 355	72 800	169 845
20	110 770	68 310	64 275	134 322
21	94 691	74 252	51 091	163 060
22	91 428	19 104	71 540	157 709
23	92 467	46 020	49 708	152 201
24	102 065	41 973	62 118	139 931
25	108 910	66 335	80 280	148 290
26	106 312	55 715	66 680	109 654
27	104 340	30 960	99 900	134 390
28	80 052	54 510	100 124	125 528
29	79 221	10 253	72 718	100 308
30	83 505	22 382	68 433	103 102
31	85 974	50 024	53 315	100 280
32	102 207	58 168	110 585	112 674
33	81 638	51 922	108 188	101 015
34	78 748	34 335	95 980	97 185
35	67 089	38 428	111 132	90 943
36	83 434	58 214	100 286	74 228
Average.....	99 693	58 843	65 006	124 886

No. 13 ran low during the entire experiment. Its maximum run was 79 613 revolutions at the sixteenth week. Its minimum run was 14 704 at the thirty-second week. Its average weekly run was 40 052 revolutions.

No. 14 showed its greatest activity from the eleventh to the seventeenth week, its maximum run being 135 316 revolutions at the fifteenth week. After that it decreased rapidly till its death in the thirty-second week. Its minimum run for an entire

TABLE 5

Number of revolutions per week of the mice in the caffeine line¹

AGE OF MICE IN WEEKS	MOUSE NO. 13	MOUSE NO. 14	MOUSE NO. 15	MOUSE NO. 16
9	27 395	25 160	51 796	51 991
10	43 205	79 935	22 685	79 617
11	41 960	102 615	33 712	143 242
12	55 474	121 164	44 478	83 853
13	34 623	87 722	44 476	141 494
14	53 403	97 081	66 508	149 307
15	47 585	135 316	64 128	128 433
16	79 613	110 157	79 980	105 980
17	48 182	104 390	46 347	112 592
18	56 228	55 084	60 028	88 598
19	51 672	82 158	58 585	103 555
20	66 583	65 340	72 885	123 895
21	53 286	66 561	58 847	87 510
22	49 292	83 704	45 256	92 468
23	58 332	61 774	48 048	72 504
24	45 728	61 374	38 114	90 872
25	35 560	26 366	47 217	111 157
26	33 015	12 512	40 410	74 635
27	33 246	4 230	43 822	70 652
28	23 602	12 657	38 868	77 981
29	24 812	451	36 690	66 117
30	18 800	1 182	29 057	44 623
31	21 940	1 007	41 248	54 748
32	14 704	721	51 550	72 710
33	22 393		59 412	66 271
34	26 256		42 240	71 520
35	26 797		41 616	77 685
36	27 790		45 855	54 098
Average	40 052	47 036	48 815	89 218

¹ No. 14 died in the thirty-second week of the experiment.

week was 451 revolutions at the twenty-ninth week. Its average run each week was 47 036 revolutions.

No. 15 ran much the same as No. 13, reaching a maximum of 79 980 revolutions at the sixteenth week. Its minimum run was 22 685 at the tenth week. Its average weekly run was 48 815 revolutions.

No. 16 was the most active of the caffeine line. It showed great activity from the eleventh to the twentieth week and rose

again at the twenty-fifth week. Its maximum run was 149 307 revolutions at the fourteenth week, and its minimum run, 44 623 revolutions at the thirtieth week. Its average weekly run was 89 218 revolutions.

Comparison of the weekly activity of all the mice

Although all these mice were of the same sex, the same age and closely related, yet even in the same lines they showed great individual variations in their activity.

Some time was necessary for the mice to get accustomed to the cages. The first week's runs are low, although the animals had been in the cages almost a week before the experiment was begun. All but one of the mice showed their greatest activity in the early part of the experiment. The decline in the latter part was probably due to increase in age. Slonaker ('07, '12) found that white rats are most active in early life.

The mice may be divided into two types, with one exception. Type 1 had a period of high activity falling within the tenth to the twenty-third week, and a second period of great activity occurring between the twenty-fourth and the thirty-second week. This would seem to be normal. This group includes six mice, controls Nos. 1, 2 and 4, nicotine Nos. 9 and 12 and caffeine No. 16. These six mice were more active than any of the others. None of them died.

Type 2 exhibited only one period of high activity which fell within the tenth to the twentieth week; the runs then decreased steadily to the end of the experiment. This includes nine mice, control No. 3, all of the alcohol mice, nicotine No. 10 and caffeine Nos. 13, 14 and 15. Only one of these, alcohol No. 7, is above the average in activity; all the others fall below the average. Three of these mice died. It is evident that these mice had less vitality than those of the first type.

No. 11 of the nicotine line is an exception to these two types. Its period of high activity did not begin until the twenty-eighth week, and its maximum run came at the thirty-fifth week.

Weekly activity of the average of each line

In order to compare the activity of the different lines the average of each line is recorded in table 6 and figure 2. Some of the mice in the alcohol and caffeine lines died during the course of the experiment. In such cases the last entire week of activity was counted in making the averages.

From table 6 and figure 2 it will be seen that the controls reached their maximum run of 119 523 revolutions at the twelfth

TABLE 6
Number of revolutions per week of the average of each line

AGE OF MICE IN WEEKS	CONTROL MICE	ALCOHOL MICE	NICOTINE MICE	CAFFEINE MICE
9	69 656	43 195	45 410	39 063
10	99 468	88 758	45 429	56 110
11	118 618	111 833	86 752	80 382
12	119 523	65 648	69 121	76 242
13	115 634	85 325	103 254	79 579
14	113 417	69 756	110 289	91 575
15	105 263	89 702	98 907	93 865
16	99 559	86 467	108 213	93 932
17	101 728	90 408	100 008	77 878
18	99 649	74 630	105 376	64 984
19	97 276	72 411	108 997	73 992
20	83 687	77 078	94 419	82 176
21	88 639	66 261	95 773	66 551
22	81 421	67 392	84 945	67 680
23	92 592	71 552	85 099	60 164
24	90 639	64 822	86 522	59 022
25	99 303	74 301	100 954	55 075
26	67 273	52 144	84 590	40 143
27	82 039	53 547	92 397	37 987
28	88 769	54 820	90 055	38 277
29	76 405	39 603	65 625	32 017
30	66 558	32 236	60 355	23 415
31	77 773	55 602	72 398	30 485
32	79 556	64 841	95 908	46 321
33	68 672	39 471	85 691	49 359
34	59 622	25 011	76 562	46 672
35	59 156	31 806	76 898	48 699
36	57 907	37 825	79 040	42 581
Average.....	87 493	63 767	86 321	59 079

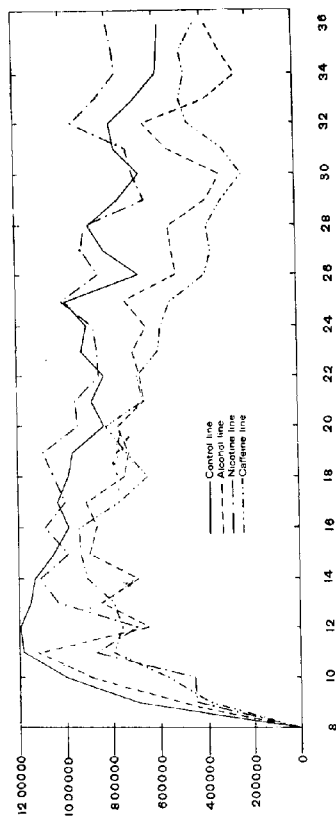


Fig. 2 Curve showing the weekly activity of the average of each line. The abscissas represent the age of the mice in weeks, the ordinates the number of revolutions.

week and then showed a rather gradual decline in running to the end of the experiment. Their last run, 57 907 revolutions, was their least. Their average weekly run was 87 493 revolutions.

The alcohol line rose almost immediately to their maximum of 111 833 revolutions at the eleventh week, but after that declined rapidly and ran low throughout the experiment. Their last run was 37 825 revolutions and their minimum 25 011 revolutions at the thirty-fourth week. Their average weekly run was 63 767 revolutions.

The nicotine line reached their maximum of 110 289 revolutions at the fourteenth week. They retained a fairly constant level with a slight decline at the end of the experiment. Their last run was 79 040 revolutions and their least, if the first two weeks are left out of account, 65 625 revolutions at the twenty-ninth week. Their average weekly run was 86 321 revolutions.

The caffeine line rose slowly to their maximum of 93 932 at the end of the sixteenth week, and showed a rather steady decline to the end of the experiment. Their last run was 42 581 revolutions and their minimum run, 23 415 revolutions at the thirtieth week. Their average weekly run was 59 079 revolutions.

The control and nicotine lines ran close together for a large part of the experiment. The controls however reached a higher maximum and reached it sooner than the nicotine mice, but after the fifteenth week the nicotine line was generally slightly ahead of the controls. These two lines correspond to type 1.

The alcohol and caffeine lines ran close together, except at the maximum of the former. After the thirteenth week they ran entirely below the control and nicotine mice. Most of the time the alcohol mice ran somewhat higher than the caffeine mice. These two lines correspond to type 2.

The alcohol mice varied more than any of the other lines. The average weekly decrease from their maximum run to their final run was 3360 revolutions, that of the caffeine mice 2566 revolutions, the controls 2566 revolutions and the nicotine mice 1420. The nicotine line kept a more constant level than the others because both No. 11 and No. 12 were very active in the latter part of the experiment.

TOTAL ACTIVITY

The total activity of each mouse is shown in tables 7, 8, 9 and 10.

TABLE 7

Total work in revolutions done by the control mice

AGE OF MICE IN WEEKS	MOUSE NO. 1	MOUSE NO. 2	MOUSE NO. 3	MOUSE NO. 4
9	80 243	78 785	55 718	63 878
10	201 178	161 313	126 759	187 248
11	323 297	279 619	225 829	327 226
12	455 890	404 620	325 829	447 721
13	594 968	545 450	406 836	549 347
14	721 323	693 026	505 802	630 119
15	845 280	824 300	587 422	714 319
16	940 150	953 495	667 450	808 464
17	1 032 748	1 073 163	752 820	917 739
18	1 141 155	1 164 467	822 404	1 047 013
19	1 228 145	1 248 567	895 504	1 191 956
20	1 294 262	1 232 554	974 248	1 297 858
21	1 368 549	1 418 201	1 032 895	1 433 836
22	1 441 439	1 482 482	1 091 014	1 564 231
23	1 523 559	1 568 437	1 167 754	1 689 803
24	1 612 013	1 661 977	1 222 299	1 757 950
25	1 719 162	1 771 492	1 276 831	1 883 968
26	1 810 884	1 872 707	1 336 891	2 000 063
27	1 904 776	1 947 187	1 390 995	2 105 843
28	1 993 531	2 057 827	1 446 175	2 206 343
29	2 060 602	2 177 208	1 487 229	2 284 356
30	2 138 041	2 240 573	1 528 110	2 368 903
31	2 226 069	2 322 173	1 575 434	2 463 043
32	2 311 414	2 407 275	1 627 598	2 568 658
33	2 384 066	2 482 100	1 663 753	2 659 715
34	2 432 446	2 554 553	1 703 578	2 737 545
35	2 506 906	2 613 873	1 744 324	2 799 645
36	2 576 101	2 677 427	1 785 571	2 857 278

The total runs are 1 785 571, 2 576 101, 2 677 427 and 2 857 278 revolutions. Three mice in this line showed great activity while one was much less active. No. 3 ran 62 per cent as much as No. 4, No. 1 ran 90 per cent and No. 2 94 per cent as much as No. 4.

TABLE 8

Total work in revolutions done by the alcohol mice

AGE OF MICE IN WEEKS	MOUSE NO. 5	MOUSE NO. 6	MOUSE NO. 7	MOUSE NO. 8
9	57 634	41 370	28 430	25 345
10	132 549	123 064	104 660	167 538
11	257 850	181 508	226 773	299 011
12	314 392	196 912	333 453	392 976
13	419 137	228 605	476 415	454 877
14	453 517	261 127	604 910	538 506
15	533 609	324 255	734 303	624 702
16	603 331	403 513	847 863	708 029
17	677 405	490 308	964 868	791 687
18	734 374	559 924	1 061 006	864 485
19	787 201	631 128	1 160 931	933 169
20	844 201	688 446	1 273 178	1 014 831
21	896 076	742 873	1 370 271	1 076 569
22	943 373	806 296	1 459 466	1 146 224
23	989 001	872 798	1 556 301	1 223 469
24	1 032 681	944 308	1 633 034	1 290 833
25	1 089 512	1 019 876	1 730 244	1 358 438
26	1 091 976	1 073 906	1 815 664	1 424 501
27	1 094 676 ¹	1 137 022	1 883 654	1 454 036
28		1 206 520	1 951 254	1 481 468
29		1 261 517	2 002 337	1 494 196
30		1 308 312	2 052 073	1 494 373
31		1 378 407	2 123 147	1 520 011
32		1 448 082	2 197 807	1 570 200
33		1 481 902	2 252 789	1 599 810
34		1 503 217 ¹	2 312 896	1 603 420
35			2 371 496	1 608 432
36			2 434 146	1 621 433

¹ No. 5 and No. 6 died at the end of the twenty-seventh and thirty-fourth week respectively.

No. 5 had run 1 094 676 revolutions at its twenty-seventh week, No. 6 1 503 217 at its thirty-fourth week, and Nos. 8 and 7, 1 621 433 and 2 434 146 respectively at the end of the experiment. The record of No. 5 was 58 per cent of No. 7 at the twenty-seventh week, No. 6 65 per cent of No. 7 at the thirty-fourth week and No. 8 66 per cent at the end of the experiment. Three of this line showed little activity, while one was much more active.

TABLE 9

Total work in revolutions done by the nicotine mice

AGE OF MICE IN WEEKS	MOUSE NO. 9	MOUSE NO. 10	MOUSE NO. 11	MOUSE NO. 12
9	25 227	36 370	44 240	75 805
10	137 513	105 118	75 458	145 272
11	271 540	158 548	112 684	267 597
12	399 014	233 969	131 751	321 989
13	506 176	332 413	146 698	514 384
14	625 344	439 110	186 910	689 436
15	747 437	533 595	220 474	834 924
16	881 052	626 870	249 962	995 398
17	1 003 622	707 148	311 194	1 131 350
18	1 127 289	784 353	381 035	1 282 142
19	1 238 279	866 708	453 835	1 451 987
20	1 349 049	935 018	518 110	1 587 309
21	1 443 740	1 009 270	569 201	1 749 369
22	1 535 168	1 028 374	640 741	1 907 078
23	1 627 635	1 074 394	690 449	2 059 210
24	1 729 700	1 116 367	752 567	2 199 210
25	1 838 610	1 182 702	832 847	2 347 500
26	1 944 922	1 258 417	899 527	2 457 154
27	2 049 262	1 269 375	999 427	2 591 544
28	2 129 320	1 323 887	1 099 551	2 717 072
29	2 208 541	1 334 140	1 172 260	2 817 380
30	2 292 046	1 356 522	1 240 702	2 920 482
31	2 378 020	1 406 546	1 294 017	3 020 762
32	2 480 227	1 464 714	1 404 602	3 133 436
33	2 561 865	1 516 636	1 512 790	3 234 451
34	2 640 613	1 550 971	1 608 770	3 331 636
35	2 707 702	1 589 399	1 719 902	3 422 579
36	2 791 136	1 647 613	1 820 188	3 496 807

The mice in the nicotine line ran 1 647 613, 1 820 188, 2 791 136 and 3 496 807 revolutions during the experiment. One showed very great activity, another great activity while two were rather inactive. No. 10 ran 47 per cent as much as No. 12, No. 11 ran 52 per cent and No. 9 80 per cent as much as No. 12.

TABLE 10
Total work in revolutions done by the caffeine mice

AGE OF MICE IN WEEKS	MOUSE No. 13	MOUSE No. 14	MOUSE No. 15	MOUSE No. 16
9	27 395	25 160	51 796	51 991
10	70 600	104 095	74 481	131 608
11	112 560	206 710	108 193	274 850
12	168 034	327 874	152 671	358 703
13	202 657	415 596	207 147	500 197
14	256 000	512 677	273 655	649 504
15	303 645	547 993	337 783	771 937
16	383 258	658 150	417 763	883 917
17	431 140	762 540	464 110	996 509
18	487 668	837 624	524 138	1 085 107
19	539 340	919 782	582 722	1 188 662
20	605 923	985 122	655 608	1 312 557
21	659 209	1 051 683	714 455	1 400 067
22	708 501	1 135 387	759 711	1 492 535
23	766 833	1 197 161	807 759	1 565 039
24	812 561	1 258 535	845 873	1 655 911
25	848 121	1 284 901	893 080	1 766 068
26	881 936	1 297 413	933 490	1 841 703
27	914 382	1 301 643	977 312	1 912 355
28	937 984	1 314 300	1 016 170	1 990 336
29	962 796	1 314 751	1 052 860	2 056 453
30	981 596	1 315 933	1 081 917	2 101 076
31	1 003 536	1 316 940	1 126 165	2 155 824
32	1 018 240	1 317 012 ¹	1 177 715	2 228 534
33	1 040 633		1 237 125	2 294 805
34	1 066 889		1 279 367	2 366 325
35	1 093 686		1 320 983	2 444 010
36	1 121 476		1 366 838	2 498 108

¹ No. 14 died at the end of the thirty-second week.

One mouse in the caffeine line showed great activity and three were much less active. Nos. 13, 15 and 16 ran 1 121 476, 1 366 838 and 2 498 108 revolutions during the course of the experiment. No. 14 ran 1 317 612 revolutions to its thirty-second week when it died. No. 13's record was 49 per cent of No. 16. No. 15 was 55 per cent and No. 14 was 60 per cent of No. 16 at the thirty-second week.

COMPARISON OF THE TOTAL ACTIVITY OF ALL THE MICE

TABLE 11

*In the following table the mice are arranged in the order of their total activity.
Total activity of all the mice in revolutions and in miles*

	MOUSE NO.	NUMBER OF REVOLUTIONS	MILES RUN
Caffeine.....	13	1 121 476	556.06
Caffeine.....	15	1 366 838	677.72
Alcohol ¹	5	1 094 676 (at 27 weeks)	542.77 (at 27 weeks)
Caffeine ¹	14	1 317 012 (at 32 weeks)	653.01 (at 32 weeks)
Alcohol ¹	6	1 503 217 (at 34 weeks)	745.35 (at 34 weeks)
Alcohol.....	8	1 621 433	803.96
Nicotine.....	10	1 647 613	816.94
Control.....	3	1 785 571	885.34
Nicotine.....	11	1 820 188	898.72
Alcohol.....	7	2 434 146	1206.92
Caffeine.....	16	2 498 108	1238.65
Control.....	1	2 576 101	1277.31
Control.....	2	2 677 427	1327.56
Nicotine.....	9	2 791 136	1383.94
Control.....	4	2 857 278	1416.73
Nicotine.....	12	3 496 807	1733.83
Average.....		2 076 622	1010.30

¹ Nos. 5, 14 and 6 died at the twenty-seventh, thirty-second and thirty-fourth weeks respectively.

From table 11 it will be seen that below the average in activity are 3 caffeine mice, 3 alcohol mice, 2 nicotine mice and one control. Above the average are 1 caffeine mouse, 1 alcohol mouse, 2 nicotine mice and 3 controls. Caffeine No. 13 was the least active of all the mice. Nicotine No. 12 was the most active. No. 13 exercised only 32 per cent as much as No. 12. No. 13 is 54 per cent and No. 12 is 169 per cent of the average.

When the revolutions are reduced to miles the average daily run of all the mice is 4.93 miles. The average daily run of the mouse that ran the least is 2.84 miles, and of the mouse that ran the most 8.99 miles. The greatest run in one day of any mouse is 16.8 miles.

*Total activity of the average of each line*TABLE 12
Total work in revolutions of the average of each line

AGE OF MICE IN WEEKS	CONTROL	ALCOHOL	NICOTINE	CAFFEINE
9	69 656	43 195	45 410	39 063
10	169 124	131 953	90 839	95 173
11	287 742	243 786	177 591	175 555
12	407 265	309 434	246 712	251 797
13	522 899	394 759	349 966	331 376
14	636 316	464 515	460 255	422 951
15	741 579	554 217	559 162	516 816
16	841 138	610 684	667 375	610 748
17	942 866	731 092	767 383	688 626
18	1 042 515	805 722	872 759	753 610
19	1 139 791	878 133	981 756	827 602
20	1 223 478	955 211	1 076 175	909 778
21	1 312 117	1 021 472	1 171 948	976 329
22	1 393 538	1 088 864	1 256 893	1 044 009
23	1 486 130	1 160 416	1 341 992	1 104 173
24	1 576 769	1 235 238	1 427 514	1 163 195
25	1 676 072	1 299 539	1 528 468	1 218 270
26	1 743 345	1 351 683	1 613 058	1 258 413
27	1 825 384	1 405 210	1 705 455	1 296 400
28	1 914 153	1 460 030	1 795 510	1 334 677
29	1 990 558	1 499 633	1 861 135	1 366 694
30	2 057 116	1 531 869	1 930 490	1 390 109
31	2 134 889	1 587 471	2 002 888	1 420 594
32	2 214 445	1 652 362	2 098 796	1 466 915
33	2 273 117	1 691 833	2 184 487	1 516 274
34	2 332 739	1 716 844	2 261 049	1 562 946
35	2 394 895	1 747 650	2 337 947	1 611 645
36	2 449 802	1 785 475	2 416 987	1 654 226

In table 12 and figure 3 the total activity of each line is averaged.

The average total activity of the caffeine line was 1 654 226 revolutions, of the alcohol line 1 785 475 revolutions, of the nicotine line 2 416 987 revolutions and of the control line 2 449 802 revolutions. The caffeine average is 68 per cent of the control average, the alcohol average is 73 per cent and the nicotine average is 99 per cent of the control average.

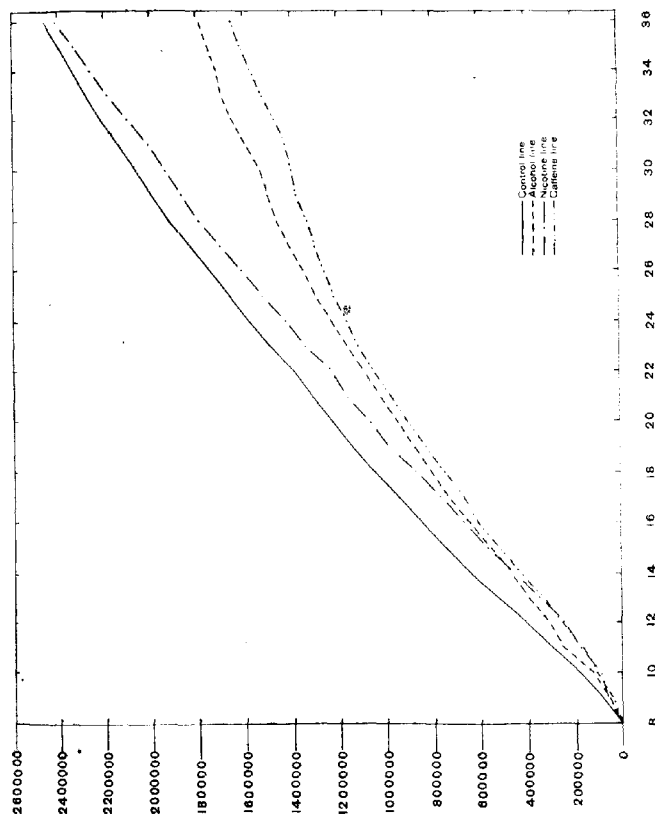


Fig. 3 Curve showing the total activity of the average of each line. The abscissas represent the age of the mice in weeks, the ordinates the total number of revolutions.

From figure 3 it will be seen that the control line leads throughout the entire time. The nicotine line excels the caffeine line at the sixth week and the alcohol line after the ninth week, and then gradually increases, keeping about an even distance from the control line, until the last two months when it more nearly approaches the control line. The alcohol average excels the nicotine line for eight weeks, but after that rises much more slowly. The caffeine average equals the nicotine average for five weeks, but after that is the lowest of all.

DISCUSSION OF THE EFFECTS OF ALCOHOL, NICOTINE AND
CAFFEINE ON THE ACTIVITY OF WHITE MICE

Control line

The control mice have the highest total activity of all the lines. They also have the highest average maximum. Three of them, Nos. 1, 2 and 4, belong to type 1, having a second period of high activity. These three are well above the average in total activity. No. 3 is less active, coming below the average. It belongs to type 2. It weighed 35 grams while the other control mice weighed 24, 24 and 25 grams respectively. Only one other mouse, caffeine No. 16, weighed as much as 30 grams. There may be some correlation between the inactivity of control No. 3 and its weight. None of the controls died. They gained on an average 7.5 grams throughout the experiment.

Alcohol line

Of the mice subjected to alcohol three are decidedly below the average in total activity, while one is slightly above it. They all belong to type 2, none having a second period of high activity, as the six most vigorous mice did. Three of them, Nos. 5, 7 and 8, start out well, but all except No. 7 rapidly decrease in activity. No. 7 seems to be an average mouse of type 2; however it loses 20 per cent of its weight during the last month. No. 5 has a maximum of 125 301 revolutions but decreases very rapidly until its death when twenty-seven weeks old. No. 6 runs low all its life, its maximum being only 86 895 revolutions. It died

when thirty-four weeks old. No 8 drops from its maximum of 131 473 regularly until the twenty-seventh week; after that its runs are irregular and some are very small. At the same time, from the twenty-eighth week to the end of the experiment it loses 30 per cent in weight. This great loss of weight and its small irregular run indicate that it would have died soon.

This line of mice started out well. They reached a high average maximum of 111 833 revolutions during the eleventh week. This is much higher than the nicotine or caffeine line at the same time but a little lower than the maximum of the control line. In fact the nicotine line never reaches quite as high an average maximum, and the caffeine maximum is only 93 932 revolutions. But after that the alcohol line drops abruptly and only once exceeds 90 000 revolutions. Their total activity is 73 per cent of that of the controls.

The alcohol mice gained 6 grams each up to the twenty-fourth week, which is more than the control mice had gained during the same time. After this, however, they lost 4.5 grams on an average. None of the other lines lost any in weight except the nicotine mice.

The activity of all the mice in the alcohol line seems to have been checked and lessened. The viability of all the mice was weakened, for two died, one was evidently going to die soon and the fourth lost 20 per cent in weight. This loss in weight occurred during the last month of the experiment. The decreased activity began to show after the mice were twelve weeks old, but the loss of weight and lessened viability did not manifest themselves until after the mice were twenty-five weeks old. Thus it appears that alcohol had a markedly injurious effect on the viability an activity of these mice and that these effects were cumulative.

Nicotine line

The mice in the nicotine line show more variations than the mice in any of the other lines. No. 10 is the least active and belongs to type 2. No. 11 is below the average in total activity. It has a very unusual record, running its least at the twelfth week

when other mice are running their highest and reaching its maximum at the thirty-fifth week when the activity of all the other mice is decreasing. No. 9 and No. 12 belong to type 1 and both exhibit great activity. No. 12 has a remarkably high record, much higher than any of the other mice in the different lines of the experiment. It exercised 169 per cent as much as the average and 122 per cent as much as control No. 4, the second most active of all the mice.

The average total activity of these mice is almost equal to that of the controls.

None of these mice died. They gained 3.5 grams during the experiment, which was less than half what the control mice gained. The last month they showed an average loss of 1.5 grams.

Nicotine did not seem to affect the health of these mice but may have slightly checked their growth.

Whether the wide variations in the activity of these mice were caused by nicotine cannot be known without further experiments. Nicotine may have had a stimulating effect on activity, shown particularly in No. 12 with its remarkable record and in No. 9. No. 10 would be an exception to such a theory. No. 11 might be explained as being naturally a very inactive mouse but that the cumulative effects of nicotine stimulated him to activity. Or it is possible that all these variations were due to chance.

Caffeine line

Three of the mice in this line have low records of total activity. Nos. 13 and 15 run very low throughout the experiment. They have the lowest records of total activity of all the mice used in the experiment. No. 13 ran 54 per cent as much as the average and 39 per cent as much as No. 4, the most active mouse in the control line. No. 14 starts out well but soon decreases in activity and dies when thirty-two weeks old. These three mice belong to type 2. No. 16 belongs to type 1 and is above the average in total activity.

The average activity of these mice is the lowest of all the lines. Their average maximum weekly run is only 93 932 revolutions

and their minimum weekly run is 24 415. Their average total activity is 68 per cent of the controls.

The caffeine mice, with the exception of No. 14, gained 11.6 grams each, which was more than any other line gained. But as they started out the smallest of all the mice and at the end of the experiments equalled the controls in weight, they apparently grew normally. No. 14 gained 3.5 grams and lost it again before its death.

The growth of three of these mice does not seem to have been affected by caffeine. One, however, seems to have been injured, for it died when thirty-two weeks old.

Caffeine appears to have decidedly lessened the activity of the mice.

SUMMARY

1. The control mice gained 7 grams on an average during the experiment. None of them died. Three were above the average in activity. Their total activity was greater than any other line.

2. The alcohol mice gained 6 grams on an average up to the twenty-fourth week, but lost 4.5 grams later. Two died and one probably would have died soon. Three were below the average in activity. Their total activity was 73 per cent of that of the controls. Alcohol appears to have had a markedly injurious effect on the viability and activity of these mice.

3. The mice subjected to nicotine gained 2 grams each on an average. None died. Two were below the average in activity and two above, one being far more active than any other mouse in any of the lines. Their total activity was 99 per cent of the controls.

Nicotine apparently did not injure the health of the mice, but seems to have checked their growth.

Nicotine may have had a stimulating effect on the activity of three of the mice. Or it is possible that the variations shown in this line were due to chance.

4. Three of the mice subjected to caffeine gained 11.6 grams each on an average, but since they started out the smallest of all

the mice and at the end of the experiment equalled the controls in weight it appears that they grew normally. One mouse died.

These mice were the least active of all the lines, their total activity being 68 per cent of that of the controls. Three were below the average in activity and one was above.

Caffeine seems to have had no influence on the growth of three of the mice, but apparently had an injurious effect on one mouse, resulting in its death.

Caffeine seems to have greatly lessened the activity of these mice.

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STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

V. THE RELATION BETWEEN RESISTANCE TO DEPRESSING AGENTS AND RATE OF METABOLISM IN PLANARIA DOROTOCEPHALA AND ITS VALUE AS A METHOD OF INVESTIGATION

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TWO FIGURES

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I. INTRODUCTION

In the preceding paper of this series (Child '12) it was shown that the dynamic processes concerned with the regulatory morphogenesis of *Planaria dorotocephala*, or at least some of them, differ in certain respects at different levels along the main axis. The evidence obtained by subjecting the regulating pieces to depressing agents and conditions suggests that the existing differences or certain of their essential factors are quantitative in nature. In fact all the results of experiments along these lines indicate the existence of a gradient in the metabolic processes or of certain fundamental processes along the main axis, the rate being highest in the anterior region and decreasing more or less regularly in the posterior direction.

But more definite evidence is necessary to determine first whether such a gradient actually exists and second, whether it is the essential or only an incidental feature of the axial factor in morphogenesis and function.

Besides the axial gradient my experiments have demonstrated the existence of various other dynamic differences depending on the age and nutritive condition and on various external factors, and in pieces on the size of the piece, the region of the body from which it came and the degree of regulation or reorganization which has occurred in it. On the other hand certain morphological characters are closely associated with certain quantitative dynamic factors.

If we can determine positively whether a quantitative factor exists in any given case and what part it plays we shall have made a real step in advance in our knowledge of development and inheritance.

The character of certain results obtained in my work on *Planaria* with depressing agents such as alcohol, KCN, etc., led me to spend much time in comparing and analyzing these results with the aid of various external and internal factors. The result of this work is the development of a method which enables us to compare in a general way the rates of metabolic reaction in different animals, pieces or regions of the body and so makes it possible to answer certain questions concerning the dynamics of organ-

isms, which have not heretofore been open to investigation. With this method we are able to determine in a general way where differences of rate exist and it is often possible also, with properly devised experiments, to determine what part these differences in rate play in phenomena of development (Child '13).

Since this method is essential for the most important results to be considered in later papers, it is necessary, before going farther in the analysis of experimental reproduction in *Planaria*, to describe the method and its application in detail. The first part of this paper is therefore devoted to a description of the method, then follows a consideration of the evidence which constitutes the foundation of the method, and finally the question as to the nature of the action of the agents used is briefly discussed and some interesting lines of investigation are pointed out.

II. THE PHYSIOLOGICAL RESISTANCE METHOD OF COMPARING RATES OF METABOLIC REACTION

1. General outline of the method

The method is concerned with the length of life, i.e., the physiological resistance of the animals or pieces in certain reagents which decrease metabolism or in sufficient concentration kill. As will appear below, a relation exists between the length of life of an animal or piece in a solution of such a reagent of given concentration under standard external conditions and the rate of metabolic reaction in the animal or piece. This being the case, it becomes possible by standardizing the concentration of the reagent used and the external conditions, to compare the rates of reaction in different individuals, regions or pieces. The present and following papers will show that this method is capable of wide application and that it gives us a new means of attack on various problems and opens up certain fields which have heretofore been inaccessible.

Assuming for the moment the correctness of the method, it is evident that we compare by means of it, not the rates of single simple chemical reactions but rather the total amounts of the reactions or processes concerned which occur in a given length of

time. This total may be made up of many individual reactions of different or of the same rate, or it may consist of a continuous reaction with variable or uniform rate. But from the amount of reaction occurring in a given time we may determine the average rate for that time. If the method under consideration is correct, it enables us to determine whether the average rate during a given time is greater or less in one case than in another. 'The rate of reaction' as the term is used here, is then analogous to the term 'rate of flow' as applied to a current of fluid. It is simply $\frac{\text{amount}}{\text{time}}$. And finally, the method in its present form is only comparative: it serves merely for the comparison of different rates without giving any information as to what the rate is in any case.

2. The relation between death and disintegration in depressing agents

The reagents most used thus far in work along these lines are ethyl alcohol and KCN. Enough work has been done with ether and chloretone to demonstrate that they give results essentially similar to those obtained with alcohol.

The first point of importance is that when an individual or piece of *Planaria* dies in a not too highly concentrated solution of any of these reagents it undergoes disintegration within a short time after death. The process of disintegration consists first in the breaking open and disappearance of the body epithelium and second of a gradual swelling and separation of the tissues until finally nothing remains but minute particles suspended in the fluid or lying on the bottom of the vessel. The swelling and increase in translucency of the tissues apparently follows almost at once after the death of the part concerned and probably results, at least in part, from the increase in permeability which occurs at the time of death. After this stage the process does not concern us so closely for it consists merely in the gradual separation of the dead cells and supporting tissues.

The length of time between apparent death and disintegration and the rapidity of disintegration vary according to temperature,

concentration of the reagent, etc., and also with various internal factors. In worms or pieces in similar physiological condition and under given external conditions, the time when disintegration begins and its rapidity are uniform to a high degree.

The close relation between death and disintegration is shown in various ways. For example, in KCN 0.001 *m.* at a temperature of 20°C. distinct movements of a given part can often be induced within fifteen minutes of the time when disintegration of that part begins. When concentrations of the anesthetics are used which are sufficiently high to kill the animals almost at once, disintegration occurs within a few minutes after the animals are placed in the reagent, sometimes beginning within five or ten minutes.

In cases where different regions of the animal or piece die at different times, we usually find certain parts of the piece still showing active movement, while others are already disintegrating or completely disintegrated.

In general then death is quickly followed by disintegration. This fact affords an easy means for determining approximately the time of death of an animal, a region of the body or a piece in a given concentration of alcohol, KCN, etc.

3. *The relation between length of life in depressing agents and rate of reaction*

Early in the course of my experiments it was found that the length of life in a given concentration of the agent used was different according to the physiological condition of the animals and in pieces, according to the size of the piece and the region of the body from which it was taken. Moreover, in different concentrations of a given reagent the relation between certain animals or pieces was not the same. At first the results appeared hopelessly complex, but I was convinced that there must be some way of discovering the factors upon which they depended and finally, after some eight months of work it became evident, first that a relation existed between the physiological resistance of the animals or pieces and their rates of reaction, and second, that the character of this relation was dependent upon the concentration of the reagent used. These relations between length of life or

resistance, rate of reaction and concentration of reagent are briefly as follows:

1. *In relatively high concentrations in which the maximum length of life is only a few hours, the length of life or resistance varies inversely as the rate of reaction: the higher the rate, the earlier death and disintegration occur and vice versa.* This form of the method which requires relatively high concentrations, I have called the 'direct resistance' method.

2. *In relatively low concentrations in which the animals remain alive for days or weeks and in which some degree of acclimatization occurs the length of life or resistance varies directly with the rate of reaction except in certain cases where incidental factors modify the result: the higher the rate, the more complete the acclimatization and the greater the length of life.* This form of the method which requires low concentrations and long times and which determines the resistance indirectly through the degree of acclimatization, I have called the 'indirect resistance' method.

3. Between these two extremes of concentration of the reagent the results vary in character with the concentration and the rate of reaction. For any two different rates of reaction it is possible to find a concentration of the reagent in which the resistance will be approximately the same: above this concentration the relation is that of the direct method, below, it is that of the indirect method.

These conclusions are drawn from thousands of experiments with alcohol and KCN by both the direct and the indirect methods. Ether and chlorotone have been used to a sufficient extent to show that with them the relations are essentially the same. Undoubtedly there are many other reagents particularly the anesthetics, which would give similar results, but since I have been primarily concerned with certain other problems, I have not as yet taken the time to test any large number of anesthetics or other substances with respect to this point. Of the different substances used, KCN has proved to be the most satisfactory. The differences in rate of reaction appear more clearly in most cases in KCN than in alcohol or other anesthetics and the concentrations used are so low that various incidental factors are

practically eliminated. From the three general rules stated above concerning the relation between resistance, rate of reaction and concentration, it is evident that care must be exercised to use concentrations sufficiently low or sufficiently high, otherwise wholly misleading results may be obtained. For example, if the concentration is too low in a test by the direct method, the animals or pieces with the higher rate of reaction may become acclimated to some extent and so may live longer than those with the lower rate which do not become acclimated to any appreciable extent. In this case the observed relation between the resistances would be the reverse of what it should be.

On the other hand, if the concentration is too high in a test by the indirect method, the animals with the higher rate of reaction may be killed by the direct action of the reagent and so die earlier than those with the lower rate of reaction, which become acclimated to some extent. Here again the results will be the reverse of what they should be.

These complications connected with the concentration on the one hand, and on the other the fact that in my earlier experiments only the indirect method, where further complications due both to internal and external factors may arise, are responsible for the long time and the large amount of work necessary for the attainment of definite results.

It is, however, a simple matter to determine the proper limits of concentration for either the direct or the indirect method. When the relation between the resistances of the animals or pieces compared does not undergo inversion with further increase of concentration, then the concentration is sufficiently high for use by the direct method and the factor of acclimatization is not involved. By decreasing the concentration from this point until the factor of acclimatization does appear clearly we can determine a concentration for use by the indirect method. In practice of course concentrations sufficiently far above or below the critical concentration are used so that there is no danger of confusing the direct and indirect effects of the reagents.

Of the two the direct method is the simpler and requires only a few hours, where the indirect method may require days or weeks

or even months. Moreover, the direct method affords no opportunity for the complication of the results by various factors which may play a part in the indirect method, e.g., starvation.

In my earlier experiments the indirect method with alcohol was used because I desired first of all to determine the effect of this and other substances on morphogenesis: the existence of the relation between length of life and rate of reaction was discovered by this method (Child '11 a, pp. 568 to 571). Later, as I became more clearly aware of the general significance of this relation, the effects of different concentrations were compared and the inversion of the relation was discovered. Later still it was found that more exact results could be obtained with KCN than with alcohol and this reagent has since been used to a large extent.

For *Planaria dorotocephala* the following concentrations have been found to be most satisfactory. For the direct method KCN 0.001 *m.* serves, although concentrations considerably lower than this may be used without altering anything but the time factor. For the indirect method very low concentrations of KCN must be used, 0.00004 *m.* or lower, i.e., acclimatization to KCN occurs only in very low concentrations.

In the case of alcohol a 4 per cent solution of absolute alcohol is commonly used for the direct method though higher concentrations may of course be used. For the indirect method a 1 per cent solution is sufficiently high when loss is prevented. In my earlier experiments, where there was some loss 1.5 per cent was used: in tightly closed flasks with very small air space this concentration is too high.

For ethyl ether 2 per cent or higher serves for the direct method and a 0.3 per cent or lower for the indirect. Chloretone has been used only for the indirect method thus far with concentrations of 0.0014 *m.* or lower.

This resistance method in general is applicable not only to *Planaria* but to any forms in which the skeleton or the connective tissue are not sufficiently developed or too closely coherent to permit the occurrence of disintegration very soon after death. I have obtained results of great interest by this method with various planarians, with coelenterates and with a number of

embryos, including those of the amphibia, and it can undoubtedly be used for many other forms. With the higher organisms the chief difficulty connected with its use lies in the determination of the time of death. If the time of death in such forms or in their parts can be determined by any other simple method than that of disintegration, there is no apparent reason why it should not be possible to compare rates of reaction by determining the physiological resistance to certain reagents of such forms or their parts.

In the following sections the practical technique of the two methods is described.

4. The technique of the direct method

In my own experiments it has proved most convenient to use lots of ten animals or pieces for each test. When larger numbers than ten are used the examination of a lot often requires too much time so that it is difficult to avoid falling behind in keeping the records. In many cases the comparison of single individuals or pieces gives perfectly definite and constant results, but the use of the larger number obviates the necessity of frequent repetition and also permits slight differences to appear which might not be discovered in the comparison of single individuals.

In the case of whole animals all ten of one lot are taken from the same stock, i.e., they have been kept in the same vessel, have received the same food and have been subjected to the same external conditions. Moreover, worms of as nearly as possible the same size are selected. In the case of pieces the ten of a lot are from animals of the same stock and the same size and the pieces are as nearly as possible of the same length and from the same region of the body. Every one of these factors is important for the result and in order to obtain definite results it is absolutely necessary that the material be standardized in this way.

When the animals are selected or the pieces cut they are usually placed as nearly as possible simultaneously or at like intervals in the required concentration of the reagent used: in certain experiments the animals must be placed in the reagent and the pieces cut in it. I have found Erlenmeyer flasks convenient except where the animals or pieces are so small that the use of a compound

microscope is necessary. In most of my experiments by the direct method 100 cc. Erlenmeyer flasks have been used. After the worms or pieces are introduced the water is poured off and they are filled with the solution to be used and corked, leaving only a small bubble of air beneath the cork to prevent bursting with slight changes of temperature. In this way loss of the substance is reduced almost to zero. Moreover, the flasks possess another great advantage: objects inside the fluid-filled flask except those on the inner surface of the glass on the side toward the observer, are magnified to a considerable extent. With a little practice the flask serves as well as a dissecting microscope and the condition of small animals or pieces can be seen very clearly.

In using the direct method, where death and disintegration occur within a few hours, we may either record only the time of disintegration, i.e., either of the beginning of disintegration or of complete disintegration, or we may follow the course of disintegration and compare different stages. Since death and disintegration occur at different times in different regions of the body the second method gives more satisfactory results: instead of recording only the beginning or the final stage, it gives a series of observations on the same material and ~~so~~ not only permits closer comparison of the different lots but increases the value of the results obtained.

In the course of my work with this method I have gradually come to distinguish five stages. The limits of each stage are of course arbitrary and some of them may, if desired, be further subdivided. These stages are as follows:

Stage I. Intact, not showing any appreciable disintegration.

Stage II. This stage is intended to record the first appearance, of disintegration in any part of the animal or piece. In whole animals the first traces of disintegration usually appear in the head region, sometimes in the most posterior zoöid. At this stage the disintegration is usually sharply localized and other parts of the body are intact and often show motor activity.

Stage III. This stage is not very sharply marked off from Stages II and IV. It is intended to include that interval between

Stage II and the time when disintegration of the marginal regions of the body is completed. In Stage III the disintegration has spread from where it first attacked the animal or piece and new areas of disintegration may have appeared; the lateral margins begin to disintegrate but the original form is still maintained. Parts of the body may still show motor activity at this stage.

Stage IV. The characteristic feature of this stage is the complete disintegration of the marginal regions and the loss of the original form which follows. The whole animal or the longer piece usually becomes more or less cylindrical, the shorter piece a rounded mass. During this stage the epithelium and pigment disappear, the dorsal surface preceding. This stage passes into the following.

Stage V. This is the last stage on which observations are made. It is reached when the epithelium and pigment are completely gone and when all parts have undergone the swelling and change in appearance. This stage I believe marks the completion of the process of dying which began in Stage II. It is followed within a short time, ranging from a few minutes to several hours, according to temperature, age of worm, etc., by separation of the tissues, disintegration of cells and gradual disappearance of the mass until all that remains are microscopic particles suspended in the fluid or on the bottom.

In whole worms, where certain regions of the body die much earlier than others, the different regions pass through the various stages at different times. The head, for example, may undergo complete disintegration before the middle regions of the body, i.e., the posterior regions of the first zooid, are dead. In recording such cases Stage II represents the first appearance of disintegration in any region, Stage III the beginning of marginal disintegration behind the head or in the posterior zooids, and Stage IV the completion of the marginal disintegration and the change in shape. Here then Stage III may be disproportionately long since different regions of the body possess different resistance. It makes little difference, however, just what each stage includes, provided it includes the same things in all cases. All that is desired is to determine as accurately as possible the time of death.

In many series with whole animals I have found it desirable to divide Stage II into two stages, II a, and II b, II a including only the earliest appearance of disintegration at any point and II b the period when the eyes and cephalic ganglia have become involved in disintegration, but other parts of the first zoöid have not yet been attacked. In such series two important periods instead of one are recorded, viz., the death of the head and the death of the last part to remain alive.

In my experiments by the direct method no attempt has been made to determine the exact time of the entrance into a given stage. That would of course be very difficult, but we may avoid the difficulty simply by examining each lot at regular intervals. At 20°C. half-hour intervals serve for KCN 0.001 *m.* and in most cases for 4 per cent alcohol. With higher concentrations or higher temperatures and sometimes with extremely small pieces shorter time intervals are often desirable and with lower concentrations and lower temperatures the time interval may be increased.

But even with this method of procedure it is of course sometimes doubtful whether a certain case should be recorded under one stage or another. My general rule in cases of this sort is to record the case under the earlier of the two stages in question: before the next observation it has passed the critical point.

In this manner then we can determine approximately the time when disintegration begins in each individual or piece and in each lot and we can also follow its course. The condition of each piece in each lot is recorded at every period of observation, i.e., commonly every half-hour and a comparison of these records brings out with much greater sharpness than a single record could the essential differences of different lots.

The record of a comparison between old and young worms is given in table 1 by way of illustration. Lot 1 consists of ten physiologically young worms 5 to 6 mm. in length, Lot 2 of old worms 18 to 20 mm. in length.

In this table the column 'Length of time' gives the length of time in the reagent in hours and minutes at each observation, the column headed 'Lots' gives the numbers of the different lots composing the series and the headings I to V under the general

heading 'Stages' indicate the five stages of disintegration. The first time given in the table shows the length of time in the reagent when disintegration was first observed in any case. The numbers in each horizontal column are the numbers of worms of each lot in each stage at each observation. The conclusion of the observations on any lot is marked by a broken line as at 4.15 for lot 1 and at 5.45 for Lot 2.

It is evident from table 1 that the young worms begin to die and disintegrate earlier and that they disintegrate more rapidly than the old worms. The results as they appear in the table are perfectly definite and clear and must have some very definite meaning.

Since the development of the direct method beyond its early stages, all the records obtained by means of it have been kept in this form. From these tables the results on any series can be seen almost at a glance.

TABLE 1

Series 557 I (Nos. 1 and 4 a). In KCN 0.001 m. 10.15 A.M., October 17, 1912

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
2.15	1	5	4	1		
	2	10				
2.45	1		6	3	1	
	2	5	5			
3.15	1		4	2	4	
	2		5	5		
3.45	1			1	1	8
	2			9	1	
4.15	1	-----				10
	2			3	7	
4.45	2				10	
5.15	2				3	7
5.45	2	-----				10

5. The technique of the indirect method

This method is chiefly useful where it is desired to follow the morphological features as well as to compare the rates of reaction. As stated on page 158 above, the results with this method are the inverse of those obtained by the direct method. There the resistance varies inversely, here it varies directly as the rate of reaction. The results by this method really represent the degrees of acclimatization to the reagent used.

In my earlier experiments, where the morphological changes were followed in animals and pieces, this method, usually with 1 to 1.5 per cent absolute alcohol as the reagent, was used exclusively. It was only after the relation between the rate of reaction and the resistance was discovered that the direct method was developed.

The data in my 'Study of senescence and rejuvenescence' (Child '11 a) were all obtained by this method. The procedure used at that time is described on pages 538 to 540 of that paper. Since then I have found it more convenient to use 1-liter Erlenmeyer flasks instead of the Stender dishes in sealed jars, as there described. The lots of worms or pieces, ten each in most cases, are placed in the flasks, which are filled with the reagent and corked, leaving an air space beneath the cork of some 10 mm. in depth. The fluid is renewed every four days or oftener, preliminary experiments having shown that in such a flask containing well aerated water twenty-five large worms would live for a week or ten days at a temperature of 20°C. without showing any bad effects. In the experiments with depressing agents the liquid is always well aerated at the time the worms are added because of the thorough shaking necessary for a uniform mixture of the water and the reagent, moreover the rate of reaction in the animals is much lower in the depressing medium than in water, so that it is impossible that lack of oxygen or harmful accumulation of metabolic products should occur at ordinary temperatures within four days.

In these experiments the lots are examined each day, or in many cases every forty-eight hours and the number of worms or pieces remaining intact is recorded. Attempts to follow stages

of disintegration are unsatisfactory here because in many cases disintegration involves certain regions, days or weeks before it does others and sometimes it involves only those regions of the body having the lowest rate of reaction, i.e., the posterior region of the first zoöid, and the body may separate into two pieces, which then undergo some degree of regulation and attain a somewhat higher rate of reaction. In other words, disintegration is often only partial and does not necessarily lead at once to the death of the whole. For these reasons it has been found best to record at each observation merely the number of individuals which remain intact.

These records can be most readily presented in graphic form as in my earlier paper (Child '11 a). Figure 1 is a reproduction of figure 2 of that paper. The starting point *a* of the curves of the axis of ordinates represents 100 per cent of the number of worms used, each small space of the cross section paper along the axis of ordinate representing 2 per cent of the total.

Along the axis of abscissae each small space of the paper represents one day. The ordinates of the various points of the curves show the percentage of worms intact at any time during the experiment, the curves being plotted from observations forty-eight hours apart.

In figure 1, the curve *ab* represents the resistance to 1.5 per cent alcohol of fifty physiologically old worms 20 to 25 mm. in length, the curve *ac*, the resistance of fifty younger worms 12 to 15 mm. in length. These results can also of course be tabulated in numerical form.

III. THE EVIDENCE FOR THE RELATION BETWEEN RESISTANCE TO DEPRESSING AGENTS AND RATE OF REACTION

1. *Resistance and stimulation*

One of the simplest ways of demonstrating the relation between the physiological resistance to a given reagent and the rate of reaction is the comparison of stimulated and unstimulated animals. Various possibilities are open here, the increase in rate of reaction following a cut or a sudden change of temperature, mechanical stimulation, etc., may with proper care be demonstrated. But

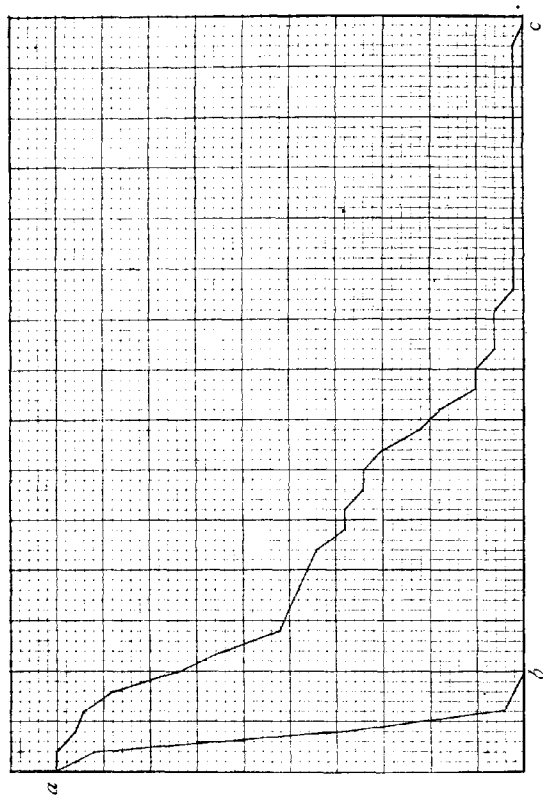


Fig. 1 Curve *ab*, the resistance of old, curve *ac*, the resistance of young individuals of *Planaria chorotocéphala* in 1.5 per cent alcohol. Each curve is based on fifty worms. Along the axis of ordinates each small space of the cross-section paper represents 2 per cent of the total number of animals in each lot; along the axis of abscissae each space represents one day.

these results will be presented in full at another time: at present only the results of a comparison between animals stimulated mechanically to motor activity and those left undisturbed will be considered. For experiments of this sort KCN is far more satisfactory than alcohol or other anesthetics since it does not inhibit motor activity so rapidly and so completely as do they. Thus far only the direct method has been used in experiments of this kind, for the indirect method would require frequent stimulation of the worms during weeks, while with the direct method this is necessary only for an hour or two.

Series 515. Two lots of ten worms each from the same stock and of the same size (18 to 20 mm. in length) were placed in 500 cc. Erlenmeyer flasks in KCN 0.001 *m*. Lot 1 was left undisturbed in diffuse daylight and the worms soon came to rest and remained almost wholly quiet until death. Lot 2 was shaken every five to ten minutes during two hours and the worms were dislodged by currents of water from a large pipette. In order to do this it was of course necessary to uncork the flask, so that some loss of KCN may have occurred in this case, but as table 2 shows any such loss certainly did not interfere with the result. The worms were examined every half-hour, but in the table only the figures for hour intervals are given.

It is evident from the table that Lot 2, the stimulated worms, begin to disintegrate before Lot 1 and continue in advance of it during the whole course of the experiment. The difference is not extreme but is sufficiently large to leave no doubt of its existence. Two of the worms of Lot 2 live as long as any of the worms in Lot 1, but the average length of life in Lot 2 is distinctly less than in Lot 1. The alternate readings omitted from the table show the same relation in every case.

That stimulation and motor activity increase the average rate of reaction, there can be no doubt and we see that the worms with the higher rate die first in the KCN. The only question which can be raised concerning these results is as to the possibility of fatigue in the stimulated lot and consequently a lower rate of reaction. This possibility can undoubtedly be excluded, for I have at various times attempted to produce fatigue by repeated

stimulation of this same sort, i.e., at intervals of five to ten minutes, and have not succeeded. Much shorter intervals or practically continuous stimulation are necessary to accomplish this result. Moreover, the actual movement in the KCN is very much less in amount than would occur with the same stimulation in water, because the animals become less and less sensitive to stimulation.

Other similar experiments have without exception given similar results.

TABLE 2
Worms placed in KCN 0.001 m. 11.00 A.M., March 8, 1912

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
2.30	1	10				
	2	8	2			
3.30	1	10				
	2	5	4	1		
4.30	1	4	6			
	2		5	5		
5.30	1		8	2		
	2		3	5	2	
6.30	1		6	4		
	2			6	3	1
7.30	1			9	1	
	2			3	4	3
8.30	1			3	7	
	2				4	6
9.30	1				5	5
	2				2	8
10.30	1					10
	2					10

2. *Temperature experiments with alcohol and cyanide*

The rate of metabolic reaction increases with rising temperature: if, therefore, my conclusions concerning the relation between resistance to certain depressing agents and the rate of reaction are correct, we should expect to find that the resistance of worms as measured by the direct method decreases with rising temperature and increases with falling temperature and this is actually the case.

But certain possible complicating factors must be considered. First there is the possibility of increased chemical activity of the reagent as a factor in the result, in consequence of the increase in dissociation at the higher temperature. In the case of KCN, however, and other substances may act in the same way, there is every reason to believe that the action is primarily upon the metabolic process at some point or points, rather than upon the relatively inactive structural substances of the organism. If this is the case the change in the rate of reaction in the organism, with a change of 10°C. in temperature, for example, must be of much greater importance in determining the observed changes in physiological resistance than the change in a 0.001 *m*. KCN solution under the same conditions. However, the evidence upon this point from the temperature experiments by the direct method alone is not demonstrative because the changes in the solution and in the organism are in the same direction. But, as will appear below, the evidence given by the indirect method in temperature experiments, as well as the evidence from experiments in various other lines leave no doubt that the rate of reaction in the organism is the chief factor in determining the results at different temperatures obtained by the direct method.

The change in the coefficient of distribution of the substances used, with change in temperature is another factor which may play a part in determining the results. According to the theory of narcosis developed by Overton and Meyer the coefficient of distribution is the most important factor in determining anesthetic action. In his third contribution Meyer ('01) shows that the coefficient of distribution of ethyl alcohol between water and olive oil increases from 0.026 and 3°C. to 0.047 at 30°C., i.e., it

nearly doubles. In the case of chloral hydrate a much greater increase occurs. Certain other anesthetics, salicylamid, benzamid, monacetin, on the other hand, show a decrease in the coefficient of distribution with rise in temperature. Meyer finds that the anesthetic action of all these substances varies at different temperatures with the coefficients of distribution. Thus the anesthetic effect of alcohol and chloral hydrate increases with rising temperature, while that of salicylamid, benzamid, and monacetin decreases.

* In Meyer's experiments tadpoles were used and the concentration of the anesthetic which would just produce complete anesthesia was determined. In other words, these results were obtained with vertebrates in which the nervous system contains large quantities of lipoid. Moreover, only the minimal concentration capable of producing narcosis was determined. It is very probable that in such an experiment with such material the coefficient of distribution is an important, perhaps the most important factor in determining the narcotic action, but it does not necessarily follow that this is the case in all other organisms. In *Planaria*, for example, there is no such accumulation of lipoids in the central nervous system as in vertebrates, and the narcotics which I have used have little if any greater effect on the nervous system than on other cells of the body, so far as can be determined. In fact it was shown in the preceding paper (Child '12 a) that development of the nervous system might go on in a concentration of narcotic which practically inhibited completely all other developmental processes.

Moreover, planarians which have been fed to repletion or during a considerable period on mammalian brain tissue do not show any decrease in physiological resistance to the action of alcohol in the concentrations used with the direct method: on the contrary, their resistance may be much greater than that of other animals which have been less heavily fed or fed on other kinds of food. The accumulation of lipoid substance in the bodies of the animals apparently does not decrease their resistance below that of animals fed on lean beef: the beef-fed animals usually show the lower rate of resistance because their rate of reaction is higher.

In the tadpole the concentration of the narcotic in the central nervous system may be greater than in other parts of the body, but there is no evidence that this is the case in *Planaria*. The assumption of the universal importance of the coefficient of distribution in the action of narcotics is not justified from experimentation of higher animals alone.

Meyer found that the coefficient of distribution of alcohol between water and olive oil almost doubled with a rise of temperature from 3° to 30°C., i.e., a change of 27°. For a change of 10° in temperature the change in the coefficient would then be comparatively slight. This brings us at once to the question whether the change in the coefficient of distribution with change in temperature is sufficient to account for the observed differences in physiological resistance. If we find that the differences in physiological resistance of two similar lots of planarians at temperatures 5° or 10° apart are considerable, we must at least admit the probability that the coefficient of distribution is not the only and perhaps not the most important factor involved. The two following series show the character of results obtained with alcohol by the direct method.

Series 561. Worms 18 to 20 mm. in length from same stock. Lot 1, ten worms in alcohol 5 per cent at temperature of 20° to 21°C. Lot 2, ten worms in alcohol 5 per cent at temperature of 10° to 11°C.

Worms in alcohol 11.00 A.M., October 29, 1912. Observations were made every half-hour but only alternate observations are given in the table.

Table 3 shows that Lot 1 at the higher temperature begins to disintegrate first and disintegrates much more rapidly than Lot 2 at the lower. In Lot 1 disintegration begins after three hours, in Lot 2 it begins in one worm after five hours, but not until after nine hours in the others. In the single case in this lot where disintegration began after five hours it remained localized in a very small area on the preocular region of the head and did not begin to advance further until after nine hours. Undoubtedly this region was a region of high rate of reaction resulting from some slight injury in handling the worms. It is possible to induce

localized disintegration by means of such slight injuries. Leaving this case out of account, the length of time to the beginning of disintegration is three times as great in Lot 2 as in Lot 1.

The worms of Lot 1 all reach Stage V within eight hours, those of Lot 2 require twenty-three hours, i.e., disintegration requires three times as long in Lot 2 as in Lot 1.

TABLE 3

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
3.00	{	1	6	4		
		2	10			
4.00	{	1		10		
		2	10			
5.00	{	1		2	8	
		2	9	1		
6.00	{	1			5	4
		2	9	1		1
7.00	{	1				3
		2	9	1		7
8.00	{	1				
		2	9	1		10
9.00		2	6	4		
10.00		2	2	8		
11.00		2		8	2	
12.00		2		5	5	
19.00		2		1	2	1
						6
20.00		2			3	1
						7
21.00		2			2	
						8
22.00		2				2
						8
23.00		2				
						10

These differences in resistance occurring at temperatures only 10° apart are certainly far greater than we should expect as the result of a change in the coefficient of distribution. With an increase of 10° the coefficient of distribution would increase less than one-third, if its increase is uniform. If this factor alone were concerned, the difference in resistance of the worms at 10° and at 20° would be slight.

Series 562 below gives results obtained with a temperature interval of only 5°C. Here young worms with a higher rate of reaction were used and also a higher concentration of alcohol, so that all times are shorter than in the preceding series.

Series 562. Worms 7 mm. in length from the same stock. Lot 1, ten worms in alcohol 6 per cent at 20°C. Lot 2, ten worms in alcohol 6 per cent at 15°C. Worms in alcohol 2.30 P.M., October 29, 1912. Observations every half-hour, table 4.

TABLE 4

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
1.30	1	3	7			
	2	10				
2.00	1		1	5		
	2	10				
2.30	1			10		
	2	7	3			
3.00	1				10	
	2		10			
3.30	1	-----	-----	-----	-----	10
	2		3	7		
4.00	2		1	2	3	4
4.30	2			1	2	7
5.00	2				2	8
5.30	2	-----	-----	-----	-----	10

In spite of the earlier beginning and the more rapid progress of disintegration the temperature difference is perfectly clear. Disintegration begins in Lot 1 after one and one-half hours; in Lot 2 at a temperature 5° lower it begins after two and one-half hours. All the worms of Lot 1 reach Stage V within three and one-half hours, while those of Lot 2 require five and one-half hours. In short, the length of time to the beginning of disintegration and to complete death (Stage V) at the lower temperature is almost twice that at the higher temperature. Differences in resistance resulting from differences in the coefficient of distribution would be scarcely appreciable with a temperature interval of only 5° , but the observed differences in resistance are almost 100 per cent. Manifestly they must be due to some other factor.

It is of interest to note that the differences in resistance in both of the above series are of the same order of magnitude as the usual temperature coefficient of chemical reaction for the temperature intervals used.

The following KCN series with a temperature interval of 13° to 15° gives essentially the same results.

Series 521 II. The worms used had been kept for three months at low temperature: during the first month it fell from 10° to 5°C. and ranged between 4° and 5° during the following two months. From this stock worms 16 to 18 mm. in length were taken for the test. Lot 1, ten worms, was placed at a temperature of 20° for twenty-four hours and then brought into KCN 0.001 *m.* Lot 2 was kept at 5° in KCN 0.001 *m.* The temperature interval is then 15° .

Worms in KCN 0.001 *m.* 10.30 A.M., March 20, 1912. Table 5 gives hourly observations.

The difference in resistance is striking. After two and one-half hours four worms of Lot 1 have begun to disintegrate, but Lot 2 does not reach this condition until after ten and one-half hours, i.e., a little over four times as long.¹ Lot 1 reaches Stage V

¹ Owing to the fact that observations were made only once an hour in this series, the earliest traces of disintegration in Lot 1 were not observed. They probably occurred about two hours after the worms were placed in KCN, but no record is made until two and one-half hours. On the other hand, in Lot 2, where disintegration proceeds much more slowly, the earliest traces are recorded at seven and

within six and one-half hours, Lot 2 within twenty-eight and one-half hours. In other words, Lot 2 requires a little more than four times as long as Lot 1 to reach a given stage of disintegration. As in the cases of the two alcohol series the differences in physiological resistance in the two lots are of the same order of magnitude as the temperature coefficient of chemical reaction for 15° . Moreover, they are certainly far greater than any possible differences in the coefficient of distribution for the temperature interval used.

And finally, the most interesting fact of all is that when the differences in resistance as expressed in the times required to reach a certain stage in the three series, 561, 562, and 521 are reduced to the same terms, e.g., to a temperature interval of 10° , they are practically identical. This fact is seen in table 6. The table shows the relation between the times required to reach given stages in the two lots of each series. For Lot 1 in each case the time is taken as unity.

The table shows that, at least for the stages considered, the rapidity of disintegration increases about three times for a rise in temperature of about 10°C . The close correspondence of the figures is all the more striking when it is remembered that in Series 561 large old worms were used, the temperature interval was 10° and alcohol 5 per cent was the reagent, while in Series 562 small young worms were used, the temperature interval was 5° and alcohol 6 per cent was the reagent, and finally, in Series 521 II large old worms were used, the temperature interval was 15° and KCN 0.001 *m*. was the reagent. This constancy of results indicates that the essential factor is in all these cases the same. The temperature coefficient of physiological resistance of *Planaria dorotocephala* to alcohol and KCN is then about 0.33 for a rise

one-half hours, but Lot 2 does not reach a stage corresponding to the first recorded stage of Lot 1, with six worms intact and four beginning to disintegrate until ten and one-half hours. Evidently then, the full difference between the two lots is given only by comparison of these corresponding stages. This gives us a relation of 1 : 4.2 while if we take the first recorded times of disintegration for both lots we obtain a relation of 1 : 3. The discrepancy between these figures is due simply to the fact that the time of the earliest stages of disintegration in Lot 1 was not recorded. There can be no doubt that the proportion 1 : 4.2 is more nearly correct than the other, 1 : 3.

TABLE 5

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
2.30	1	6	4			
	2	10				
3.30	1		7	3		
	2	10				
4.30	1			8	2	
	2	10				
5.30	1				6	4
	2	10				
6.30	1	-----				10
	2	10				
7.30	2	9	1			
8.30	2	9	1			
9.30	2	8	2			
10.30	2	6	4			
11.30	2	5	5			
12.30	2	2	6	2		
13.30	2		8	2		
* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *
20.30	2			4	6	
21.30	2			3	7	
22.30	2			2	6	2
23.30	2			2	6	3
24.30	2				7	3
25.30	2				6	4
26.30	2				5	5
27.30	2				3	7
28.30	2	-----				10

TABLE 6

The relation between the resistances at different temperatures in Series 561, 562 and 521 II, reduced to a temperature interval of 10° C.

	BEGINNING OF DISINTEGRATION		STAGE V	
	Lot 1	Lot 2	Lot 1	Lot 2
Series 561.....	1	3.0	1	2.8
Series 562.....	1	3.33	1	3.33
Series 521 II.....	1	2.8	1	2.9

in temperature of 10°C. The fact that this coefficient is the reciprocal of the temperature coefficient of chemical reaction, together with the fact that other lines of experiment show that the resistance depends upon the rate of reaction, justify the conclusion that this temperature coefficient of resistance is essentially dependent upon the rate of reaction in the planarian body.

Nevertheless, when substances whose coefficient of distribution increases with rising temperature are used with the direct method, the possibility remains in temperature experiments that the coefficient of distribution may be an important factor in certain species or certain organs rich in lipoids. It should be possible, however, to show whether it or the rate of reaction in the organism is the important factor in a given case by using some of the depressing agents which show a decrease in the coefficient of distribution between water and fat with rising temperature. If we should find that in spite of the lower coefficient of distribution the resistance of the animals was less at higher than at lower temperatures, or even the same at both temperatures, we should be forced to conclude that the coefficient of distribution was not the essential factor in determining the result. This question will be considered in the following section.

The results of temperature experiments by the indirect method also afford further evidence bearing upon the point in question. By the indirect method with alcohol as reagent the resistance is less at lower and greater at higher temperatures. It is evident that only the differences in the rate of reaction in the organism can be responsible for this result. The animals become acclimated to the reagent less readily and less completely at the lower than at the higher temperature.

On pages 564 to 568 of the paper on senescence (Child '11 a) the records of such a temperature series were given. Figure 2 is a reproduction of figure 15 of that paper. The series (Series 140) was prepared as follows (Child '11 a, pp. 565-6):

The worms used were 15-18 mm. in length and were well nourished when collected (Nov. 25). They were kept in the laboratory 24 days at a temperature of 18° - 22°C . without food, during which time they used up most or all of their reserves. At the end of this period a stock of about 100 worms was placed in dishes surrounded by running water at a temperature of 8° - 10°C . From this stock two sets of 10 worms each were taken after 12 days, 22 days, 37 days and 65 days at the low temperature. One of these sets in each case was placed in alcohol 1.5 per cent at the same temperature, 8° - 10°C ., at which the worms had been kept, the other in alcohol 1.5 per cent at room temperature, 18° - 22°C .

The two curves in figure 2 are plotted from these two lots of forty worms each. Since each lot of forty worms is made up of worms taken at different periods during sixty-five days, the curves do not show the changes in resistance which occurred in the stock during this period, but since these are the same at any given time for all the worms of the stock, it is better for the present purpose to eliminate them. The two curves then show simply the effect of placing worms which had been kept in water at a certain temperature, in alcohol at different temperatures. The lower curve *ac* shows the resistance of the worms at the lower temperature, the upper curve *ab* the resistance at the higher temperature. As in figure 1, each small space of the cross section paper along the axis of ordinates represents 2 per cent of the total number of worms used, and each space along the axis of abscissae represents one day. The curves show that the worms at the higher temperature possessed greater resistance.

In another similar series the stock was kept at room temperature, 18° to 22°C . and from this parallel lots were taken at intervals, one lot in each case being placed in alcohol at 18° to 22°C ., the other in alcohol at 8° to 10°C . In this series, as in Series 140, the worms in alcohol at the lower temperature always showed less resistance than those at the higher.

There can be no doubt, I think, that the difference in resistance under these conditions is due to the difference in the rate of reac-

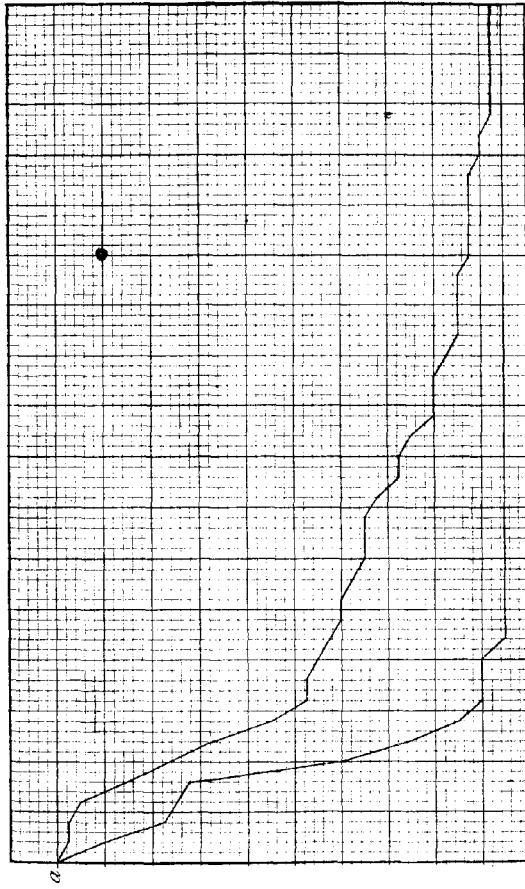


Fig. 2 The resistance of *Planaria dorotocephala* in 1.5 per cent alcohol at different temperatures. Each curve is based on forty worms. Curve *ab*, the resistance at the higher, curve *a* the resistance at the lower temperature. Along the axis of ordinates each small space of the cross-section paper represents 2 per cent of the total number of animals in each lot; along the axis of abscissae each space represents one day.

tion in the organism at the different temperatures. If the coefficient of distribution were the most important factor, the resistance would be lower at the higher temperature. As a matter of fact, the animals with the higher rate of reaction determined by the higher temperature become more readily and more completely acclimated and therefore live longer.

Taken as a whole, the evidence from temperature experiments with alcohol and KCN for the existence of a relation between physiological resistance and rate of reaction is highly conclusive. Within certain limits, the higher the temperature, the higher the rate of reaction, the less the resistance by the direct method, and the greater the resistance by the indirect method.

How far these relations will hold for the higher animals, and particularly those in which the nervous system contains a large amount of lipoid material, can be determined only by experiment. At present I can state that they hold for all flatworms that have been tested, some five species, for *Corymorpha palma* among the coelenterates and for early embryonic stages of various species, so far as tested, including the amphibia.

3. *Temperature experiments with benzamid*

On page 179 above, attention was called to the bearing upon the question of the relation between resistance and rate of reaction of experiments with a substance whose coefficient of distribution between water and fat increases as the temperature falls. In temperature experiments with such a substance the animals should die earlier at the lower temperature if the coefficient of distribution or some other factor similarly affecting its concentration in the cell is the chief factor in its effect, but if the rate of reaction in the organism is the chief factor, then the animals should die earlier at the higher temperature in spite of the lower coefficient of distribution at this temperature.

The coefficient of distribution of benzamid as determined by Meyer is 0.672 at 3°C. and 0.437 at 36°C. (Meyer '01, pp. 341-344). In accordance with this difference in the coefficient of distribution, Meyer found that the minimal concentration which would produce complete narcosis in tadpoles was $\frac{1}{500}$ m. at

3°C. and $\frac{1}{200}$ m. at 30°C., i.e., the narcotic effect was greater at the lower temperature. In his experiments Meyer determined merely the minimal concentration that would produce complete narcosis and apparently did not attempt to determine what would occur with higher concentrations.

My own results with different concentrations are as follows: with concentrations near or somewhat above the narcotic minimum (viz., 0.02 m.) the narcotic effect is greater and disintegration occurs earlier at the lower (10–11°C.) than at the higher temperature (20–21°C.) With concentrations considerably higher than this, on the other hand, the temperature relations are just the reverse; the narcotic effect is greater and disintegration occurs earlier at the higher than at the lower temperature. With these higher concentrations of benzamid the temperature relations are the same as with the higher concentrations of alcohol and KCN and with the lower concentrations they are the same as with the lower concentrations of alcohol and KCN. *In spite of the higher coefficient of distribution at the lower temperature, the lower rate of reaction in the organism at this temperature determines with higher concentrations of benzamid a higher resistance than at the higher temperature where the rate of reaction is higher.*

The question at once arises as to how far the coefficient of distribution and how far other factors are concerned in these relations. When low concentrations of alcohol and KCN are used the animals with the higher rate of reaction become more readily and more completely acclimated and so die later than those with the lower rate. But such concentrations of alcohol and KCN are below the minimum which produces complete narcosis. In the case of benzamid the narcotic effect with minimal concentrations is greater at low and less at high temperatures. It is certain that the acclimatization factor is not involved in this primary narcotic effect. There are, however, indications that acclimatization to benzamid occurs very rapidly; in sufficiently low concentrations the animals show signs of recovery from the partial or complete narcosis in less than twenty-four hours at 20°C. In disintegration experiments, therefore the acclimatization factor may play a part as it does with alcohol and KCN, but the

temperature relations of the primary narcotic effect must be due to some other factor.

Since the temperature coefficient of distribution of benzamid changes with change of temperature, in the opposite direction from that of alcohol the next step is to determine how far this factor is responsible for the observed results.

When the concentration of the solution is below a certain limit the benzamid does not enter the cell with sufficient rapidity or in sufficient quantity to produce any appreciable physiological effect; it may be oxidized or disposed of in some other way as rapidly as it enters. Assuming that the substance enters the cell through the lipoids, it is evident that with certain low concentrations the presence or absence or the degree of the narcotic effect may depend on the coefficient of distribution. With such concentrations, the higher the coefficient of distribution, the higher the concentration of benzamid in the cells and the greater the effect. On this basis we might interpret the greater narcotic effect of the benzamid and the lower resistance of the worms at the lower temperature when concentrations near the narcotic minimum are used, as due to the higher concentration of the substance in the cell at the lower temperature in consequence of the higher coefficient of distribution.

But when concentrations considerably above the narcotic minimum are used it is evident that in spite of differences in the coefficient of distribution, within a wide range of external conditions the concentration of the substance in the cell is sufficient in all cases to produce complete narcosis and sooner or later death. In such concentrations the coefficient of distribution must become a factor of minor importance and the rate of reaction in the organism is the chief factor. If these conclusions are correct, it is evident that the coefficient of distribution is not the essential factor in narcotic action in any case. The lipoids may be largely responsible for the entrance of the narcotics, at least in certain cases, but their effect after they have entered is apparently primarily chemical. This conclusion is in essential agreement with the most widely accepted theory of narcosis.

With a closer scrutiny of the temperature experiments, however, certain obstacles to this simple interpretation appear. If

the temperature coefficient of the rate of chemical reaction in the organism is of the same order of magnitude as that found for many other chemical reactions then the rate of reaction in the organism must increase two to three times with a rise of 10°C . On the other hand, according to Meyer, the coefficient of distribution decreases only about one-third with a rise of about 30°C . In short the temperature changes in the rate of chemical reaction are so much greater than those of the coefficient of distribution that it is difficult to understand why the first factor does not overbalance and mask the second. For example, in 0.02 *m*. benzamid the animals are narcotized both at 10° and at 20° , but more rapidly at 10° and they also die earlier at 10° . Evidently at 10° enough benzamid enters the cell to produce the full physiological effect. At 20° the coefficient of distribution is only very slightly lower than at 10° , but the rate of reaction in the organism is supposedly two to three times as great as at 10° . The temperature coefficient of distribution of benzamid for 10°C . is so small that it cannot possibly account for the observed results which are constant and distinct.

There can be little doubt, I think, that with sufficiently low concentrations of benzamid the increase in rate of reaction in the organism is the chief factor in determining that the narcotic effect is less at the higher than at the lower temperature. At 10° the rate of reaction is so low that the concentration of benzamid in the cell is sufficient to produce the full physiological effect: at 20° the concentration in the cell as determined by the coefficient of distribution is only very slightly lower, but the rate of reaction in the animal is now two to three times as great as before, i.e., it is now so great as compared with the concentration of benzamid in the cell that only a fraction of the total reaction volume can be affected by the benzamid, consequently the physiological effect is less at the higher than at the lower temperature.

And here the factor of acclimatization enters. We have seen that for KCN and alcohol, the higher the rate of reaction, the greater the degree of acclimatization, provided the concentration of the reagent is sufficiently low. The same rule holds good for benzamid. In this case it is impossible to determine where the

primary effect ends and acclimatization begins, for both show the same relation to temperature changes and one merges into the other.

But if we accept the above interpretation of the results obtained with *Planaria*, how are we to interpret Meyer's results with tadpoles, viz., that in temperature experiments the narcotic effect varies with the coefficient of distribution. It is evident that if only minimal concentrations of the reagents are used, if the volume of lipoids in an organism or tissue is very great and if the total volume of chemical reaction is relatively small, then the coefficient of distribution may become the chief factor in determining the physiological effect of a narcotic. In the vertebrate nervous system the volume of lipoids is very great and, except in the earlier stages of development, the actual volume of chemical reaction is small. Moreover, Meyer's results are for minimal concentrations only. Undoubtedly the concentration of the narcotic in the nervous system of the tadpoles is the chief factor in determining the narcotic effect. It is at once apparent that this is a case where the coefficient of distribution may be the determining factor, but it is also apparent that generalization on the basis of this case alone can lead only to wrong conclusions. If Meyer had worked with some of the lower invertebrates as well as with the tadpoles and if he had used higher as well as minimal concentrations, he would have reached very different conclusions.

My experiments permit only the conclusion that the action of benzamid on *Planaria* is of essentially the same character as that of alcohol, KCN, etc. In higher concentrations the resistance of the animals varies inversely as the rate of reaction; in lower concentrations it varies directly as the rate of reaction, except in certain cases where incidental factors such as the coefficient of distribution, nutritive condition, etc., play a part. Under the usual conditions and in organisms where the differentiation of tissues, and especially the accumulation of lipoids is not very great the rate of reaction in the organism is by far the most important factor in determining the resistance and the other factors become practically negligible. In general then the experiments with

benzamid afford a complete confirmation of the conclusions based on the work with alcohol and KCN.

The records of the two following series show the results with a concentration considerably above the minimum (Series 566) and with a concentration near the minimum (Series 564).

Series 566, A 1, B 1. Worms 8 to 9 mm. in length, all from the same stock. Lot A 1, ten worms at 21°C.; Lot B 1, ten worms at 11°C. Condition recorded every fifteen minutes, table 7.

The worms used here were rather small and young, with a relatively high rate of reaction. Lots A 2 and B 2 of the same series consisted of very large old worms: in those disintegration proceeded much more slowly, but the worms at the higher temperature died first, as in table 7. In other series similar results were obtained.

The table shows clearly that Lot A 1 disintegrates more rapidly than Lot B 1, although the coefficient of distribution is lower in the case of Lot A 1. On the other hand, it is evident that the difference in rate of disintegration between the two lots is very much less than in alcohol and KCN series with the same temperature interval. At first glance, the obvious conclusion seems to be

TABLE 7
Worms in benzamid, 0.04 m. at 12.00 noon, November 15, 1912

LENGTH OF TIME	LOT	STAGES				
		I	II	III	IV	V
1.15	A 1	2	7	1 ⁵		
	B 1	10				
1.30	A 1			8	2	
	B 1	10				
1.45	A 1			3	5	2
	B 1			10		
2.00	A 1				4	6
	B 1				10	
2.30	A 1	-----	-----	-----	-----	10
	B 1				6	4
2.45	B 1	-----	-----	-----	-----	10

that the two factors, coefficient of distribution and rate of reaction balance each other to some extent and that since the rate of reaction has the greater temperature coefficient it determines the result. Undoubtedly this is to a certain extent correct, but it is difficult to understand how the relatively small temperature coefficient of distribution can so nearly balance the much greater temperature coefficient of rate of reaction. I am strongly inclined to believe that another factor is involved here. The macerating effect of benamid is very great; the tissues seem almost to dissolve in it. High concentrations of alcohol produce the same effect to some extent and it appears to a greater extent in ether. It is probable that disintegration in high concentrations of these and many other substances which are highly fat-soluble is not solely the result of the narcotic action, but in part of a change in physical condition in consequence of the solution of the substance in the lipoids. The cells and tissues are undoubtedly dissolved to some extent. This physical effect apparently hastens disintegration and often decreases the differences due to different rates of reaction. With KCN this factor is eliminated for all practical purposes, if it exists at all in that case. The concentrations of KCN used are so very low that everything except the chemical factor disappears from the result. For this reason results obtained with substances which must be used in high concentration or which are very highly fat-soluble should always be checked by KCN.

The concentration of benamid used in Series 566, viz., 0.04 *m.*, is more than double the minimal narcotic concentration for either of the temperatures used. This concentration is near saturation at 10°C., so that higher concentrations can be used in temperature experiments only when higher temperatures are used. But with higher concentrations the physical factor undoubtedly becomes still more important, so that we should expect the differences due to rate of reaction in the organism to become less and less marked with increasing concentration.

For comparison with Series 566 another series is given in which the concentration used was lower, 0.02 *m.*; this is only slightly above the minimum.

Series 564, A 1, B 1. Worms 8 to 9 mm. in length, all from the same stock. Lot A 1, ten worms at 20° to 21°C. Lot B 1, ten worms at 10° to 11°C. Only hourly observations recorded. Table 8.

Observations were not carried further on this series. Table 8 shows two gaps in the observations, one during the night, when no observations were made, the other after the death of B 1 where the records are omitted from the table as not essential. These gaps do not interfere in any way with the definiteness of the results.

Here the worms show earlier and more rapid disintegration at the lower temperature: apparently some other factor than the rate of reaction is the chief factor here. Here again the temperature coefficient of distribution is certainly not sufficient to account for the marked difference between the two lots. As a matter of

TABLE 8
Worms in benzamid, 0.02 m. 11.45 A.M., November 11, 1912.

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
4.15	A 1	10				
	B 1	6	4			
5.15	A 1	10				
	B 1	4	6			
6.15	A 1	10		•		
	B 1		10		•	
7.15	A 1	10				
	B 1		5	5		
8.15	A 1	10				
	B 1			8	1	1
* * * *	* * * *	* * *	* * *	* * *	* * *	* * *
21.15	A 1	3	3	2	2	
	B 1				4	6
22.15	A 1	3	2	2	2	1
	B 1					10
* * * *	* * *	* * *	* * *	* * *	* * *	* * *
29.15	A 1		1		1	8

fact, these results correspond to those obtained by the indirect method with alcohol and KCN: there is no doubt that a certain amount of acclimatization occurs in Lot A 1 of this series. At 22.15 hours the three worms of Lot A 1 which were still intact showed some slight recovery from the complete narcosis of the preceding day. In other words, this concentration of benzamid gives us indirect results in which the acclimatization factor is* involved, consequently here the resistance of the worms varies directly as the rate of reaction.

These two series are sufficient to show that the coefficient of distribution of benzamid is of little importance in determining the narcotic effect on Planaria and the resistance of the animals to it. Here as in the case of alcohol, the rate of reaction is the most important factor. It must not be forgotten, however, that in the vertebrates with the great volume of lipoids in the nervous system the coefficient of distribution of a narcotic may be a much more important factor in determining its physiological effect. But there as elsewhere, this factor remains a condition, not a cause.

4. The evidence from animals of different age

When young and old animals are compared by the direct method the resistance of the young animals is always much lower than that of the old. This method has been used with both KCN and alcohol on Planaria dorotocephala and with KCN on P. maculata, P. velata, Phagocata gracilis, Mesostomum sp. and embryonic and larval stages of Amblystoma. In all cases the same result was obtained, the younger animals died and disintegrated earlier than the older. In the cases of Planaria dorotocephala and P. velata both the younger and older animals were undoubtedly the products of asexual reproduction, as sexual reproduction has not been observed in P. velata and only in a single individual in P. dorotocephala during the years that I have had these forms under observation. In Planaria maculata, on the other hand, the young worms used in experiment were raised directly from eggs laid in the laboratory and in the other forms mentioned above asexual reproduction does not occur. As a

matter of fact, there is no fundamental difference, though there may be a difference in degree between planarians asexually produced and those arising from eggs. The following series gives characteristic results for *Planaria maculata*.

Series 51 II and VI. The younger worms (Lot 1) were 3 to 4 mm. in length and had emerged from egg capsules in the laboratory during the last few days preceding the experiment; they had been fed with earthworm once, two days before the test was made.

The older worms (Lot 2) had hatched several weeks earlier and had been fed with earthworm until they had attained a length of 8 to 9 mm.

Each lot consisted of ten worms. Table 9 gives the data in the same form as the preceding tables. In this series observations were made every fifteen minutes, but since the results at all stages are perfectly uniform and definite, only the alternate readings are given in the table.

In Lot 1 disintegration begins earlier than in Lot 2 and all the worms are completely disintegrated within two hours after being

TABLE 9
Animals in KCN, 0.001 m. 1.35 P.M., September 1, 1912

LENGTH OF TIME	LOTS	STAGES				
		I	II	III	IV	V
1.00	{ 1 2	6 9	4 1			
1.30	{ 1 2			5 2	2	3
2.00	{ 1 2	----- 2	6	1	1	10
2.30	2		5	3	1	1
3.00	2			4	3	3
3.30	2			1	3	6
4.00	2				1	9
4.30	2	-----	-----	-----	-----	10

placed in the KCN, while the worms of Lot 2 remain intact in most cases longer than those of Lot 1 and require four and one-half hours to reach Stage V.

That the rate of reaction is higher in the more recently hatched worms cannot be doubted. They are more active and grow more rapidly than the older worms and there is not the slightest reason to doubt that if we could measure their metabolism directly, as has been done for higher animals, we should find that they, like the young of higher animals, show a higher rate of reaction per unit of body weight than the older animals.

The following simple experiment also indicates that the rate of reaction in the young worms is higher than that in the old. If a miscellaneous stock of several hundred worms, including both young and old is placed in water in an Erlenmeyer flask, which is then tightly corked, the worms begin to die within a few hours and it is always the young worms which die first. That death in this case is due to lack of oxygen rather than to the presence of CO_2 or other products of metabolism is indicated by the fact that the water from such a flask in which worms are dying rapidly will not kill other worms, provided they have access to a small bubble of air. The effect of KCN 0.001 *m.* is almost exactly similar to that of lack of oxygen: in both cases the worms with the higher rate of reaction (the young worms) die first.

The possible objection that the smaller size of the younger worms may in some way determine the result is met by the following facts: the younger worms do not simply disintegrate faster than the older, they begin to disintegrate earlier; it is difficult to see how difference in size alone can account for this difference. Secondly, in certain experiments on nutrition to be described elsewhere, the larger worms disintegrate earlier than the smaller, because of a higher rate of reaction resulting from a different nutritive condition. In fact a large number and variety of experiments to be described will demonstrate beyond a doubt that size alone is a factor of comparatively little importance. It is, in fact, one great advantage of the method that it is at least very largely independent of size.

All results obtained thus far from experiments by the direct method on animals of different ages, whatever the species, are essentially similar to those of table 9. On page 165 the records of a series with asexually produced young and old individuals of *Planaria dorotocephala* are given to illustrate the method of recording data. There also the young animals begin to disintegrate earlier and disintegrate more rapidly than the older.

When we compare these results with the other lines of evidence it is clear that all are in essential agreement. The only possible factor that can be responsible for the observed differences in physiological resistance is the rate of reaction in the organism.

In the paper on senescence two series are presented showing the differences between young and old animals from nature by the indirect method (Child '11 a, pp. 544-547, figures 1 and 2; figure 2 is reproduced as figure 1 of the present paper). Other figures show similar differences produced in a variety of ways: for example, figures 3 to 7 of that paper show differences in resistance, i.e., in rate of reaction produced by differences in nutrition and figures 9 to 14 show how pieces may become physiologically young as the result of regulation.

By the indirect method the animals with the higher rate of reaction show the higher resistance, viz., they become more readily and more completely acclimated.

5. Further miscellaneous evidence

Further evidence for the existence of a relation between physiological resistance and rate of reaction is obtained from various other lines of experimentation which will be considered fully in other connections.

By means of the direct method it is possible to distinguish the change in rate due to various forms of stimulation. For example, a piece isolated by cutting has, during the first few hours after the section, a much lower resistance, i.e., a much higher rate of reaction, than the same region of the uninjured body. This method shows further that the resistance of such pieces gradually

increases, namely, their rate of reaction decreases during the first twenty-four hours after section, until, except in relatively large pieces, it is greater (i.e., the rate of reaction is lower) than that of the corresponding region in the uninjured animal. In such a sequence of events we see first the sudden rise in rate due to the cutting; after this the rate gradually falls as the effect of the cutting gradually decreases, until finally the rate in small pieces is lower than when they formed part of the uninjured whole. This low rate of small pieces as compared with the uninjured whole, is, as I shall show later, a result of isolation, viz., of the absence or decrease in the action of physiological correlative factors which before isolation played an important part in maintaining the average rate of reaction at a certain level. Manifestly, such changes in physiological resistance occurring in an isolated piece within twenty-four hours or less, cannot be readily or consistently interpreted on any other basis than that of the rate of reaction, especially when they are compared with results obtained in other ways.

Moreover, it can be shown by the same method that the decrease in resistance (i.e., the increase in the rate of reaction in pieces following cutting) can be largely prevented by partial anesthesia at the time of cutting.

And finally, as regulation proceeds in the piece, the resistance gradually decreases, namely, the rate of reaction gradually rises, until in cases where the piece forms a new whole, the resistance becomes much lower, i.e., the rate becomes much higher than it was originally when the piece was a part of the uninjured animal. In short, the new whole is physiologically, as well as morphologically, younger than the part of the animal which it originally represented.

Again, the change in resistance in a piece as compared with the corresponding region of the uninjured animal, varies with the degree of mutilation, viz., the rate of reaction increases temporarily as the degree of mutilation increases. And the greater the amount of regulatory reorganization in a piece, the lower the resistance as measured by the direct method, becomes; in other words, the higher its rate of reaction.

Turning to another line of experiment, we find that in pieces of a given size from a given stock of worms, the different types of head show different resistances by the direct method. In general the normal head shows the lowest resistance, that of the teratophthalmic head is somewhat higher and the increase continues through the teratomorphic and anophthalmic to the headless type (for a description of these different types of head, see Child '11 b '11 c). I have already shown (Child '11 c '12 a) that we can induce the appearance of the more abnormal types of head in place of the less abnormal by decreasing the rate of reaction in the head-forming regions and can bring about changes in the opposite direction by increasing the rate of reaction in this region. According to the results of the direct method of determining the resistance, the normal head shows the highest rate and from this the rate decreases through the various forms to the headless type. Here then the two lines of experiment—production of the abnormal types of head with the aid of external factors and determination of the physiological resistance of the different types—are in complete agreement and we cannot doubt that the difference in resistance in the different types is determined by the differences in rates of reaction.

It remains now to mention a widely different line of evidence which serves to confirm the results of the resistance method. Dr. Tashiro, an assistant in Dr. A. P. Mathews' laboratory at the University of Chicago, has recently devised an exceedingly delicate apparatus which makes it possible to determine and compare the amounts of CO_2 -production in small or nearly quiescent organisms or pieces of tissue. Dr. Tashiro has been kind enough to make a number of comparative tests of CO_2 -production in individuals and pieces of *Planaria* under different conditions; in every case, the results obtained are parallel to my own, obtained with KCN and alcohol. Animals or pieces which by the direct method show a lower resistance show in this apparatus a higher rate of CO_2 -production. Direct comparisons by means of this apparatus are limited to animals and pieces of approximately the same size, so that as far as my own results concern animals and pieces of different size they cannot be directly confirmed in this way.

Thus far comparison of the rate of CO_2 -production has been made between relatively long anterior and posterior pieces, between short anterior and posterior pieces, between animals in different nutritive condition, between early and later stages of regulation and between moving and quiescent animals and in every case the result obtained paralleled that obtained by the direct method. Moreover, in almost every case the result was obtained without knowledge of the result which I had obtained with the resistance method. I am under great obligation to Dr. Tashiro, both for his kindness in making the tests and for permission to make this statement.

These data obtained by a totally different method from my own afford a most valuable confirmation of the results of the resistance method. The only possible conclusion is then, I believe, that the resistance of *Planaria*—as well as of various other forms—to certain reagents is in general a measure of the rate of metabolic reaction and can be used as a basis for comparing the rates of reaction of different animals and pieces under different internal and external conditions.

6. The value and the limitations of the resistance method

It is evident that so long as disintegration is the criterion of death, this method can be used only in cases where death is followed within a short time by disintegration. So far as my experience goes, this occurs only in those forms where a highly differentiated connective tissue or a well developed skeleton is absent. For example, the method gives very definite results in the earlier stages of amphibian ontogeny, but by the time the animals hatch, the skeleton and connective tissue have attained a consistency such that disintegration does not occur for days after death. If we can find some other satisfactory criterion of death, we can of course apply the method much more widely. Failing this, it may be possible to use the indirect method to some extent in such cases, for with that method determination within a day or two of the time of death is in most cases sufficient. As yet I have not attempted to develop the method along this line.

For those forms where disintegration follows soon after death, the direct method is of much greater value than the indirect. In the first place, it is more accurate and permits the determination of smaller differences in rate of reaction. With the direct method the rate of reaction during the first few moments after the worms are placed in the reagent, or at most an hour or two, is the chief factor in determining the time of death. With the indirect method the rate of reaction during days or weeks is a factor in the results, but during this time the rate may change in consequence of gradual starvation, or in pieces in consequence of regulatory processes, moreover, external conditions, e.g., temperature) may also alter the rate and so influence the result. To take a case in point, suppose we compare large relatively old worms with very small young worms. By the direct method the young worms show a much higher rate of reaction than the old, but by the indirect method the factor of starvation may give the results a wholly misleading character; in other words, the very small worms with a much higher rate of reaction may, in spite of this rate, die of starvation before the large old animals, with much lower rate, die from inability to become acclimated. Nevertheless, it is of interest to note that except in certain extreme cases of this kind the smaller, younger animals live longer, even though they have less material available for nutrition. It is perhaps possible that in alcohol, with which most of my work by the indirect method was done, these younger worms with their higher rate of reaction are able to make some use of the alcohol as a nutritive substance. This complicating factor is of course absent when KCN is used.

If a concentration of alcohol near the limit at which acclimatization becomes impossible, is used, the temperature factor may appear very clearly: after the animals have been in the solution for some time and are less vigorous than normal, a rise in temperature of a few degrees may change the action of the reagent from what I have called the indirect to the direct and the animal dies.

All of these and doubtless other factors also, interfere with the accuracy of the indirect method, but in the direct method they can be eliminated with little difficulty. It is always desirable to check by the direct method results obtained by the indirect.

There are also in certain cases regional factors which make the application of the indirect method difficult. When, for example, the rate of reaction in different parts of the body is very different it is sometimes difficult to find a single concentration which will give results by the indirect method for both.

In animals like many of the coelentera and turbellaria the continued existence of the characteristic structural features after they have once developed is more or less directly dependent on the maintenance of a certain relatively high rate of reaction. Decrease in the rate below a certain limit is followed either by dedifferentiation or more usually by death. It is difficult to compare rates of reaction by the indirect method in such cases for the direct depressing effect of the reagent often kills or hastens the death of certain relatively highly differentiated parts, even when the concentration is so low that it has little or no effect on other parts. The hydranth region of the hydroid *Corymorpha* is a case in point. In all my tests by the indirect method the hydranth region dies before the stem, although young hydranths live longer than old ones in the same solution. These cases need further investigation, but apparently we have here simply a structure in which the range of acclimatization is narrowly limited by the high degree of differentiation which has resulted from the relatively high rate of reaction. It will probably be found true in general that structural differentiation, especially where it occurs in consequence of a high rate of reaction, limits the range of adaptation to depressing media. Moreover, the older such a structure becomes the more narrowly is its range limited. In *Tubularia*, for example, the mere change from open water to the laboratory usually brings about the death of the hydranths, apparently in consequence of the decrease in rate of reaction accompanying the change in conditions: use of a depressing agent hastens the death of the hydranth still further because it brings about a still further decrease in the rate of reaction.

And finally there are the factors of time and labor to be considered in connection with the indirect method. New solutions must be made up every day or two and the renewal of the solutions in the flasks requires much time. The direct method requires only

one supply of solution and observations extend over only a few hours.

But although the indirect method is much less valuable than the direct for the comparison of rates of reaction alone it is of great value in the analysis of morphogenesis for it enables us to determine with some degree of certainty the relative rates of reaction connected with different morphogenetic processes and to inhibit the processes with lower rate (Child '12). Moreover, I believe it will prove of value as a method for the experimental study of acclimatization. Certain points of considerable interest have already appeared in the course of my work with this method, although I was chiefly concerned with other problems. For example, the fact that old animals always die at a larger size than young ones in certain low concentrations of alcohol must have a very definite physiological significance. Under these conditions the old animal is not able to use as large a proportion of its own substance for nutrition as is the young animal. Possible interpretations of this fact will be considered elsewhere.

As regards the relative value of different reagents, there can be, I believe, but one conclusion, viz., that the cyanides are far more valuable than any of the others. The very low concentrations which are used, as well as the constitution of the cyanides practically eliminate various factors which may complicate the results obtained with the narcotics in the stricter sense and leave only the chemical factor. The results obtained with the cyanides must, I think, be taken as the basis of the method and other results must be checked by them.

With pure reagents, water of constant constitution, constant temperature, care in making solutions and the proper care of stocks and selection of animals the direct resistance method is a method of great delicacy and the complicating factors which influence the results by the indirect method can be practically eliminated in the direct method. The method is undoubtedly capable of much further development as an exact method than I have yet attempted. By standardizing the conditions of the experiment and adopting a certain unit as a basis of measurement we may obtain definite dynamic expressions for different ages,

different conditions of nutrition, different degrees of regulation, etc.

It is of course possible that further use of the method may bring to light further complicating or limiting factors but it is certain that the method is capable of very wide application. But in addition to the immediate results the method gives us a means of attack on various problems which have not heretofore been open to investigation.

During the last few years I have used both the direct and the indirect methods in a very large number of experiments and with a variety of forms. The results obtained afford a new insight into the dynamics of living organisms, they throw light on the problem of physiological polarity and symmetry, they afford a dynamic basis for the law of antero-posterior development and they have demonstrated that changes in the rate of metabolic reaction may bring about changes in the number, localization, degree of differentiation, etc., of definite morphological characters. Besides this they have made it possible to demonstrate the essential similarity between the process of regulation by which a piece gives rise to a whole and the other forms of reproduction and development in nature. They have given us a new viewpoint from which to consider the questions of senescence and rejuvenescence. And finally, certain of the results obtained have a very direct bearing upon the problem of inheritance. Some of the more important results have already been briefly presented (Child '12 b) and these and others will be more fully considered in following papers.

IV. THE ACTION OF DEPRESSING AGENTS IN GENERAL

1. *The nature of the action of depressing agents on Planaria*

It is not my intention to discuss at length the problem of narcosis, but merely to call attention to certain points.

The poisonous effect of the cyanides is very generally regarded as due, at least in large part, to a retardation or inhibition of some sort of the oxidation processes. According to Geppert ('89) they render the tissues incapable of uniting with oxygen.

Loeb and various others have used the cyanides extensively during later years as a means of retarding or inhibiting the oxidation processes.

It is also certain that the planarian in KCN shows a decrease in CO_2 -production as compared with a normal animal.

At any rate, it is evident that the CN radical affects some of the most fundamental metabolic reactions. Moreover, the fact that the effect of the cyanides varies according to the rate of reaction in the organism suggests that the action is primarily chemical. Apparently the effect of a KCN-solution depends upon the number of chemical bonds in the organism which are opened up in a given length of time; in other words, the higher the rate of reaction, the greater the opportunity for the KCN to produce its effect.

Apparently the KCN acts by entering the metabolic complex at some point or points and altering certain essential features of it so that it cannot continue. That the point or points of entrance lie somewhere along the course of the oxidation processes, is the most generally accepted view.

But how are we to conceive the process of acclimatization to the cyanides? It occurs only in very low concentrations, but that it does occur there can be no doubt. Why does the relation between the capacity for acclimatization and the rate of reaction exist, i.e., why does the individual or piece with the higher rate of reaction become in general more readily and more completely acclimated? It is evident that a metabolic factor is involved in the process of acclimatization, but we are at present far from any real knowledge as to the nature of the process. The relation between the rate of reaction and the degree and rapidity of acclimatization must have a very definite meaning and it may perhaps serve as a basis for further work along this line.

We have seen that with alcohol, ether, chloretone and benzamid essentially the same relation between physiological resistance and rate of reaction in the animals exists. Since my investigations have had thus far another object, I have not as yet attempted to determine the relations for any very large number of substances, but the occurrence of the same relation between resistance and

rate of reaction with all the substances used indicates clearly a certain similarity in the nature of their action.

Moreover, the effects of alcohol, ether and chloretone upon morphogenesis are in their essential features similar to those produced by KCN and on the other hand to those produced by low temperature, products of metabolism in the water, etc. (Child '11 c '11 d). Evidently all these substances act in some way to decrease the rate of the metabolic processes. Similar conclusions have been reached by various authors and on the bases of various lines of investigation. The similarity in the effects of KCN and alcohol, ether, etc., suggests that all act in some way on the oxidation processes.

But that the action of the alcohol is not the same as that of the cyanides is clearly indicated by the difference in the capacity for acclimatization to the two substances. Alcohol 4 per cent kills the worms within a few hours, but in alcohol 1 per cent most worms become acclimated. KCN 0.001 *m.* kills the worms in about the same time as alcohol 4 per cent, but no appreciable degree of acclimatization occurs in concentrations higher than 0.00004 *m.* To what is this difference due?

The planarian is exceptionally good material in many ways for determining the physiological effect of chemical substances, and particularly for purposes of comparison of different substances. In many cases also two different aspects, the physiological and the morphological, of the effect may be compared with each other: this possibility in turn gives us a method of attack on certain morphological problems which have scarcely been accessible heretofore.

The results of the experiments with depressing agents on *Planaria* have an important bearing on the general theory of narcosis for they indicate very clearly that the coefficient of distribution of the narcotic between water and fat has no necessary relation to its narcotic action. In *Planaria*, where there is no great accumulation of lipoids in any organ, we have seen that the coefficient of distribution is a factor of very little importance as compared with the rate of reaction in the organism. But attention has already been called to the point that in the vertebrates, where the

accumulation of lipoids in the nervous system is very great, the coefficient of distribution may be a very important factor in determining the concentration of the narcotic in the nervous system and so in determining its physiological effect. But it is evident that in such cases the fundamental relation between the physiological effect of the narcotic and the rate of reaction in the organism or organ is simply masked by the incidental factor of coefficient of distribution.

2. Certain differences in the action of different reagents

Notwithstanding the general similarity in the physiological and morphological effect of the different substances used thus far, certain more or less characteristic differences exist.

In the first place, there is a marked difference in the relation between mortality and morphological effect. In alcohol, ether, chlorotone a considerable proportion of the pieces die in any concentration high enough to produce a marked morphological effect. In KCN, on the other hand, it is possible to obtain extreme morphological effects without losing a single piece in large series of several hundred pieces. Such a difference as this must have some very definite meaning.

But certain other minor differences appear to be more or less characteristic of the different substances. For example, KCN inhibits or retards the formation of the optic pigment to a greater extent than any other reagent used. In many cases eye-spots consisting only of the unpigmented areas are formed (Child '12, pp. 124-125). The outgrowth of the auricles is also greatly retarded or completely inhibited in KCN, although the characteristic unpigmented sensory area develops in the same manner as when the auricle grows into its normal form. In general the consistency and color of the new tissue formed in KCN differs to some extent from that of tissue which develops in other reagents.

Ether apparently inhibits the development of new tissue at the cut surface to a very great extent: in some cases a new head, or at least a ganglionic mass may develop in ether with scarcely a trace of outgrowth from the cut surface (Child '12, pp. 120-

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121). Thus far, this condition has been observed only in ether. In alcohol, on the other hand, some development of new tissue occurs in any concentration which does not kill.

The morphological effect of chloretone is very similar to that of alcohol but appears with much lower concentrations (alcohol 1–1.5 per cent; chloretone 0.02–0.025 per cent).

As already mentioned above (p. 188) a much greater degree of maceration and apparent solution of the tissues occurs in alcohol, ether, chloretone and benzamid than in KCN; this may be due to the different effects of these substances on the lipoids.

The effects of low temperature are in general similar to those produced by the substances mentioned, but in low temperature the growth of new tissue is merely retarded, not inhibited. The relation between the formation of new tissue at the cut surface and the redifferentiation back of the cut is much the same as under the usual conditions. In this respect low temperature differs in its action from all the substances used.

Thus far I have not been able to discover any features strictly characteristic of the animals which develop in water containing an excess of their own metabolic products. Conditions of this sort which produce marked morphological effects usually produce also a high mortality as is the case with alcohol and the morphological effects resemble those of alcohol.

More attention directed to this point will doubtless bring to light other morphological or physiological effects characteristic of the different substances. At present, however, there seems to be no ground for believing that any of these features which are more or less characteristic of one or the other substance are specific chemical effects. Some of them may conceivably be such, but it is much more probable that they are the result of incidental physical factors, e.g., osmotic conditions, coefficient of distribution, etc. In general the morphological effects of these substances and conditions are due primarily to differences in the rate of reaction in different developing organs and regions.

In more highly differentiated animals than *Planaria*, where the difference in constitution in different organs is much greater than here it should be possible to produce morphological effects with

features more distinctly characteristic of the different reagents. The slight differences of this sort in Planaria are, I believe, good indications of the low physiological specification of the tissues.

V. SUMMARY

1. In Planaria and in many other forms in which there is no highly specialized skeletal or connective tissue, death is followed within a short time, varying from a few minutes to a few hours, by disintegration. This fact makes it possible to determine with some degree of accuracy the time of death of animals, regions of the body or pieces.

2. Experiments with stimulated and unstimulated animals, with animals at different temperatures, with old and young animals, with animals in different nutritive condition and with pieces of different size, from different regions of the body and under different conditions demonstrate the existence of a relation between the length of life (physiological resistance) of the animals or pieces in KCN, alcohol and various other anesthetics and the rate of the metabolic reactions or certain of them, probably the oxidations.

3. In relatively high concentrations, in which the maximum length of life is only a few hours, the length of life (resistance) varies inversely as the rate of reaction in the animals or pieces. The higher the rate of reaction, the earlier does disintegration begin and the more rapidly it proceeds and vice versa. This is the direct method of comparing rates of reaction.

4. In relatively low concentrations, in which the animals remain alive for days or weeks and in which a greater or less degree of acclimatization occurs, the length of life (resistance) varies directly as the rate of reaction, except in certain cases where incidental factors contribute to the result. The higher the rate of reaction in the animal the more complete the acclimatization, at least in most cases, and the greater the length of life. This is the indirect method.

5. With concentrations between these two extremes the results differ according to the concentration of the reagent used and the

rate of reaction in the organism. For any two rates of reaction it is possible to find a concentration of the reagent in which the resistances will be approximately the same. In order to avoid misleading results it is necessary to be certain that with the direct method the concentration is sufficiently high and with the indirect method, sufficiently low.

6. These two methods, and especially the direct method, afford a means of attacking various problems and they serve particularly to give us some insight into the dynamics of morphogenesis.

November, 1912.

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THE REACTIONS OF FISHES TO GRADIENTS OF DISSOLVED ATMOSPHERIC GASES

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I. INTRODUCTION

The importance of atmospheric gases to aquatic animals has been coming more and more to the attention of biologists through the work of Marsh and Gorham ('05), Marsh ('10), and Reuss ('10) on the effect of gases upon fishes; through the studies of reversal of behavior reactions by varying amounts in solution (Loeb '04, Mast '10, Wodsdalek '11, and Allee '12); and through the survey of the distribution of gases, which has been carried on in the Wisconsin lakes by Birge ('04, '07a, '07b, '10) and by Birge and Juday ('11). The effect of the different gases, or of varying amounts of a particular gas upon animals has been but little studied. There are many facts concerning the distribution of aquatic animals which do not seem to be explainable on the basis of amount of oxygen considered as a life and death matter (Shelford '11 b, and '11 d); the junior author experienced difficulty in the control of gases in solution in connection with his study of isopod behavior. We accordingly decided to design gas control apparatus and to study the reactions of some group of animals to gas gradients, making it a joint investigation with important bearings upon our separate interests. Fishes were selected because their physiology, habits, and distribution are well known.

II. ATMOSPHERIC GASES

1. OCCURRENCE

The chief facts concerning the occurrence of gases in nature and their solubility under experimental conditions are shown in table 1. The standard method of expressing quantity of gas in solution is in cubic centimeters per liter at 0°C. and 760 mm. of mercury. All values are therefore given in these terms.

The amount of each gas that will go into solution from the atmosphere or under experimental conditions, is determined by its solubility and partial pressure and by temperature. The relative solubility of the atmospheric gases is indicated in table 1, by relative amounts going into solution at 20°C. and 760 mm. of mercury. If we desire to increase the total amount of gas in

solution we must either increase the total pressure, decrease the temperature, or substitute a more soluble for a less soluble gas.

TABLE 1 •

Showing the distribution and solubility of atmospheric gases

GAS	COMPOSITION OF AIR IN PER CENT OF TOTAL	GAS VALUES IN CC. PER LITER AT 0°C. AND 760 MM. MERCURY			KIND OF WATER HAVING GAS CONTENT GIVEN IN PRECEDING COLUMN
		At temperature 20°C. 760 mm. mercury		Maximum amounts found in natural fish waters; springs ex- cepted	
		Water absorbs from air	Water absorbs pure gas		
Nitrogen, etc.....	79.02	12.32	15.00	19	Lakes; Birge and Juday (‘11, p. 52)
Oxygen.....	20.95	6.28	28.38	24	Streams, lakes in winter, and with green algae
Carbon dioxide.....	0.03	0.27	901.00	30	Ponds
Ammonia.....	small traces locally		very large quantities	14	Sewage contaminated; Nichols (‘04, p. 62)
Methane.....	small traces locally		34.00	10	Bottom of lake in Sept.; Birge and Juday ‘11, p. 101

2. EXPERIMENTAL CONTROL OF GASES IN SOLUTION

The two methods commonly employed in the control of gases in solution in water are (a) The reduction of gas content by boiling or by vacuum pumps, and (b) The increase of some one gas by bubbling it through water.

a. Gases used

The analyses of the commercial gases were made with the Hempel ('02, chap. 3) apparatus. The carbon dioxide was absorbed with $\frac{53}{3}$ per cent potassium hydroxide; the oxygen with yellow phosphorus except when it constituted more than 50 per cent of the total, in which case alkaline pyrogallol was used. Oxygen and carbon dioxide in solution were determined by the methods given by the Committee on Standard Methods of Water Analysis of the American Health Association ('05, pp. 72-77), and by boiling. Boilings were made in a boiler like that described by Birge and Juday ('11, p. 7), which holds two liters. The gas obtained was analyzed by absorption and was corrected

for temperature, pressure and tension of aqueous vapor (Hempel '02, p. 64). The results of the analyses are shown in table 1 A.

TABLE 1 A

Analyses of commercial gases used in experiments

GAS	ANALYSES, IN PERCENTS OF TOTAL			SOURCE	DEALER
	Oxygen	Nitrogen	Carbon dioxide		
Oxygen.....	99.0	0.95	trace	Liquid air	Chicago Calcium Light Co.
Carbon dioxide.....		0.6	99.4	Coke ovens	Liquid Carbon Dioxide Co.
Nitrogen.....	7.5	92.5		Liquid air	Linde Air Products Co.

b. Hydrogen, ammonia and methane

No experiments were performed with hydrogen. Shull ('12) has found that hydrogen manufactured electrolytically and on sale by the Lind Air Products Company commonly contains from 2 to 4 per cent of oxygen. Hydrogen which is generated in the usual way and used for the purpose of displacing oxygen, probably always contains a quantity of oxygen. The ammonia used in these experiments was the chemically pure solution diluted and allowed to flow into the apparatus at a uniform rate. The effect of methane upon animals has probably not been studied. Crocker and Knight ('12) manufacture it and have found that it slightly affects plant growth.

c. Bubbling gas through water

Gas was bubbled through water in order to learn something of the cause of gas bubble disease (Marsh and Gorham '05) which developed in the stock of fish while the experiments were being conducted. Under these conditions, not one but several factors were varied. The gas which is bubbled through is added to the water and each bubble being a partial vacuum for the other gases, takes them up until they are nearly exhausted from the water. The results of some of the bubbling experiments are

TABLE 2

Showing the effect of bubbling gas through a square vessel 30 cm. in depth, holding four and one-half liters of water, and with 150 sq. cm. exposed to the air. The gas was introduced at the bottom. Where not given, average flow was about 50 cc. per min.

WATER USED	GAS USED	GENERAL CONDITIONS			CARBON DIOXIDE		OXYGEN		NITROGEN	N ₂ SAT. AT T	O ₂ SAT. AT T	TOTAL ON BOILING
		Time in hours	Flow in cc. per min.	Temp. in deg. C.	Titration	Absorption	Titration	Absorption				
Aqua dist.	exposed to atmosphere	21		18.5	3.0	1.1	6.8		12.9	13.1	6.75	
Tap water.	N ₂	20		19.0	0.0	1.1	3.5	5.5	14.5	12.5	6.40	21
Aqua dist.	N ₂	24	30	18.5	0.5	1.27	4.6			12.6	6.47	18.8
Aqua dist.	O ₂	24	168	18.5	1.5	0.3	25.8			12.6	6.47	25
Tap water.	CO ₂	27	180	18.5	662.7		1.1			12.6	6.47	600
Aqua dist.	CO ₂	54		19.0	495.9	493.1	0.4		6.3	12.5	6.40	500

shown in table 2. The flows of gas as given are averages as the tanks were not supplied with valves giving constant flows and it was necessary to adjust the flows every few hours.

The second test given in the table shows that exposure to the atmosphere, necessitated by filling the boiler with sufficient water to allow it to run through only once, made accurate determination impracticable, with the apparatus at hand. We note that when oxygen is bubbled through water, the oxygen supply is not only increased but carbon dioxide and nitrogen are decreased. When distilled water is used the carbon dioxide is lowered, but when tap water is used, if the tap water contains bicarbonates, the bicarbonates are largely changed to carbonates (McCoy and Smith '11, McCoy and Test '11), the water becomes alkaline and a slight cloud sometimes appears. This indicates that some carbonates are precipitated and free and half-bound carbon dioxide are both removed just as in boiling. This changes the number of alkaline metal ions in solution. The general results of bubbling nitrogen through tap and distilled water are respectively similar except that nitrogen is increased instead of oxygen. When carbon dioxide is bubbled through, it may reach 662 cc. or more if the flow is rapid. All carbonates are kept in solution; oxygen is greatly reduced. Loeb ('04, p. 7), Mast ('11, p. 179) and Wodsedalek ('11, p. 270) bubbled carbon dioxide through

water and ascribed the results to the carbon dioxide though all the gases were affected. Loeb in his experiments on Amphipods, says that the water had an acidity equal to M/500, which is about 44.8 cc. per liter. None of the others give carbon dioxide determinations. The presence of fishes modifies the results of bubbling.

TABLE 3

Showing the results of bubbling gas through jars of water containing fishes, the jars being 50 cm. high, with an exposed surface area of about 43 sq. cm. and holding about 2.2 liters. The gas was introduced at the bottom. The nitrogen was not determined because of the small quantity of water. Average flow 50 cc. per min.

EXPT.	GAS		FISH			TEMP.	OXY- GEN	CARBON DIOXIDE	TOTAL GAS ON BOILING
	Kind used	Flow in cc. per min.	Time bub- bled, in hours	No.	Length in cm.				
I	O ₂	43	43	5	5-15	20	11.9		
II	O ₂		23	2		20	16.5	4.6	27.5
III	N ₂		54	2	7-10	20	2.1	2.5	22.7
IV	N ₂	54	24	2	7-10	19.5	4.4	2.0	19.5
V	N ₂		23	4	5-10	19	0.5		
VI	CO ₂		5	2	8	19	1.1	163.0	
VII	CO ₂		2	2	8	19	4.6	153.0	
VIII	standing water			4	5-10	20	1.1		

d. The reduction of gas content and the addition of particular gases

A laboratory water supply is usually supersaturated with gases when it comes from a body of water exposed to the atmosphere. It is often unsuitable for animals because of its low oxygen and high nitrogen content when it comes from wells (Marsh '10). In the first case the animals suffer ill effects from the excessive amount of gas, while in the second they suffer from the deficiency of oxygen. Either of these difficulties may be remedied by passing the water through a series of perforated vessels or allowing it to flow in thin sheets over large rough surfaces (Marsh '10). To reduce the gas content to a point below saturation, requires either the use of a vacuum pump or boiling. In either case all the gases are affected and it is often necessary to add certain gases in order to obtain the desired conditions. The exhausting

of gases by pumps or boiling, offers no difficulties if only small quantities of water are desired but if a continuous flow is needed the difficulties are greatly increased. The exhausting of gases with pumps is to be preferred, as it interferes less with the plankton, the bicarbonates; it would, however, no doubt remove some of the half-bound carbon dioxide and thus affect the bicarbonates present in solution to some extent.

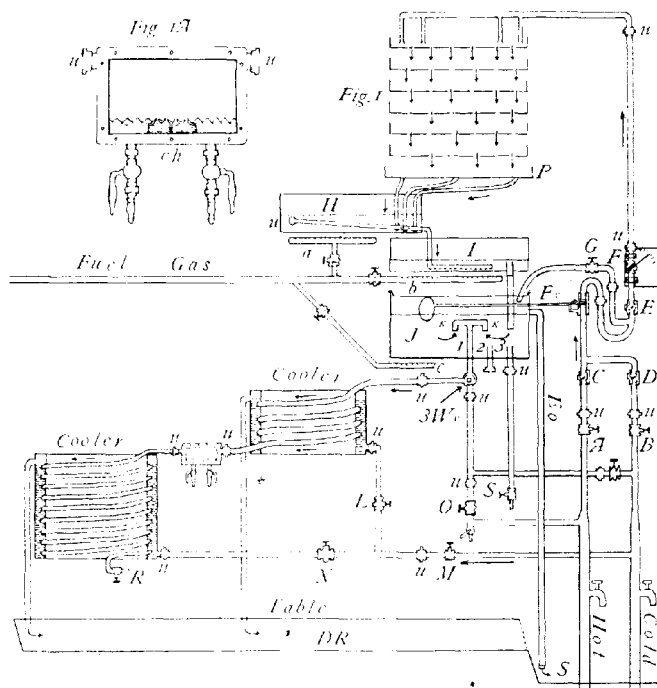
A piece of apparatus to give a constant flow of water from which the gas has been exhausted by a vacuum has been devised but a good air exhaust and compression pump and considerable experimentation would be necessary to perfect the first machine and as yet this has been found impracticable. Apparatus which boils water and gives a constant flow is less expensive, particularly because less skilled labor is required to build it. A combined atmospheric deaërating and boiling device installed for the control of gases is shown in figure 1. The principle is as follows: The hot water of the laboratory system is passed through a float valve of high grade, which regulates the flow into a storage or delivery tank. Above the storage tank and between it and the float valve, are two open boilers, which bring the water to the boiling point, and a series of sieves which lowers the gases to saturation when the boilers are not used. The water is withdrawn from the storage tank through two tap water coolers. When the boilers are used, the water comes off with a very low gas content.¹

With the aid of figure 1 we may follow the course of the hot water through the apparatus when a flow of water at cold tap temperature is desired. First the water passes through valve *A* to the float valve *Fv* which is opened by the lowering of the water in the receiving tank. The water then passes through the 'return bend' to valve *F* which is set so as to give about the flow desired. After passing this valve the water rises to the top of the appa-

¹ The cost of such a piece of apparatus varies greatly with the conditions and methods of installation. The total cost of the present piece, including labor, sink, drain and small water table, was about \$300. The plans were drawn and all the parts ordered by the authors, who also supervised the installation. \$500 to \$600 would be a fair contract price in Chicago if detailed plans were furnished.

ratus and is distributed on the topmost sieve. From there it flows through the series of sieves to the collecting pan *P*. From here it is conducted to boiler *H* by means of several small aluminum tubes which reach to the bottom of the boiler and thus introduce the water as near the heat as possible. The water is conducted from boiler *H* through a large pipe attached to its

Fig. 1 Apparatus for the control of gas content of water. For description of the course of the water, see text, p. 213. The water supply pipes are $\frac{1}{4}$ inch reduced to $\frac{3}{8}$ inch above the branch going to the cooler from the cold water pipe and to the three way valve from the hot. The valves *A*, *B*, and *M*, are $\frac{1}{2}$ inch straight way valves. Above valves *A* and *B* are swing check valves *C*, *D* to prevent the water's backing into the coolers when a mixture is used. The float valve is one manufactured by L. Wolf Manufacturing Company, with ground brass union soldered to the delivery tube. The shape of the valve makes necessary a



return bend of $\frac{3}{4}$ inch galvanized iron, with a third swing check valve (*E*) inserted at the level of the float valve, to hold the weight of the water in the pipe, as the float valves are not constructed so as to withstand back pressure. The valve *F* is a tee handled valve with a brass bar 7 inches long bolted to the tee, a piece of metal plate clamped to the pipes, lies directly behind the brass bar and the position of the bar for 1200 cc. and 3600 cc. per minute under average pressure is marked on the plate. Four $\frac{1}{2}$ inch iron pipes are used to distribute the water over the top sieve. The sieves are 18-inch garbage-can lids, with about 1,200 1 mm. holes punched in, 1 cm. apart. The collecting pan is of galvanized iron, being also a garbage-can lid, a little larger than those mentioned above, with $8\frac{1}{2}$ inch holes. Aluminum tubing $\frac{1}{2}$ inch inside diameter is threaded into iron nuts, which are soldered to the lid directly beneath the holes.

The boilers, *H* and *I* are aluminum saute pans without handles, purchased from the Aluminum Cooking Utensils Company. They are drilled and the drain pipes are lock nutted into position. The pipe leading from the upper to the lower boiler is of galvanized iron. The small perforated pipe is of brass. The delivery tank is of galvanized iron but for durability an aluminum vessel should have been used. The gas burner *a* is a water heater burner; *b* is made up of six small unit burners, so that the amount of heat may be fully controlled; *c* is a gas range burner which keeps the water in the delivery tank at the boiling point. The delivery tank is supplied with an emergency overflow (*EO*). The withdrawal tubes *K* and *K'* of the delivery tank are $\frac{1}{2}$ inch galvanized iron pipe, which fits the smallest size three way valve (3 *W*); (for withdrawals 2 and 3, see text). The first cooler contains 15 feet of block tin pipe $\frac{3}{8}$ inch inside; the second cooler contains 60 feet of block tin pipe, $\frac{1}{2}$ inch inside. The tin pipes are soldered to brass fittings. The valve (*R*), at the final outlet, is a $\frac{1}{2}$ inch straight way valve. The coolers are supplied with tap water through $\frac{1}{2}$ inch iron pipe attached with unions. Valves *O*, *N*, and *L*, are $\frac{1}{2}$ inch brass gas service cocks with tee handles. The coolers are made by setting one galvanized iron tank into another, the pipe being fastened to the wall of the inner tank. The overflows of the tank lead to a lead covered trough which empties into the sink (*S*). The inner space in the lower tank, is intended to be used as an ice pack in summer when a temperature lower than the tap water is desired.

The gas introducer *A*, is a brass chamber (*cm.*) with half unions on the ends, and a plate glass front pressed against rubber packing by means of screws and a brass frame. On its lower side are two small chambers (above *C'h*), which are connected with the main chamber by very small holes drilled in the apices of small cones hammered into the metal. Each small chamber communicates with a cock which may be attached to a tank of gas. The pipes in the two coolers are connected with the gas introducer by one-half unions. Many brass ground joint unions, wherever practicable, make possible the detachment of any part. The entire apparatus occupies a space above the table 97 by 130 cm., the highest part reaching to the ceiling. It is supported on a frame of iron pipe made by screwing together cut pipe and fittings described on pages 188 A-189 A, of Crane Company's catalog No. 40. The sieves are supported on wire pins placed in holes drilled through both walls of the four pipes which make the tower. Because of the small space available and because the completed apparatus is the result of several experiments, there are unnecessary complications.

side to the bottom of boiler *I*. In this boiler, the water usually reaches the boiling point before flowing into the receiving tank. It flows out through double downward curved tubes *K*, to the cooling coils under the pressure of a column of water of the height of the delivery tank. The curves in the pipe in the coolers cut down the pressure particularly in the lower cooler where the pipe is $\frac{1}{16}$ inch, so that the flow from the outlet valve cannot exceed 1200 cc. per minute. The treated water may be at the exact temperature of the tap water and may contain as little as 0.3 cc. of oxygen per liter. If the water in the delivery tank falls below the lower ends of the withdrawal tubes *K* and the coils fill with air, the water does not flow. To clear the pipes of bubbles, the hot or tap water may be forced through them by turning the three-way valve beneath the delivery tube and opening valve *O* or the corresponding tap water valve.

At all times of the year, but especially in the winter and spring, the tap water from Lake Michigan is supersaturated with gases, and in an open vessel the gases come off in bubbles which soon cover the bodies of animals in experiments, and thus render their activities abnormal. Fish kept in aquaria, which are supplied with this water, die within a few days or even hours, from gas disease. To remedy this and to supply water which is free from excess gas, cold water is allowed to run through the apparatus and is withdrawn from either the second or the third withdrawal. The second withdrawal is supplied with a union by means of which iron pipe may be attached to the aquarium in which fish are kept, while the third is supplied with a hose-end near the surface of the water table. With this apparatus we have been able to secure water of any desired temperature and any desired gas content, nitrogen excepted, within the limits and needs of our problem.

(1) *Effect of the apparatus upon the water.* Here we are concerned with three things: (1) The normal tap water, (2) the hot water, and (3) the water that has passed through the apparatus.

(1) The cold water supply is from the Chicago water system which uses water taken from Lake Michigan at a distance of $2\frac{1}{2}$ miles from shore and from a depth of 6 feet where the water

is 90 feet deep. (2) The hot water is from the same general supply but passes through a heater into which live steam is introduced. It comes from the tap in the laboratory, where the apparatus is installed, at a temperature of from 40 to 60°C. and is highly supersaturated with gases; this water was used for boiling.

A large number of metals were used in the apparatus, principally because the materials were at hand, but it was also thought that the use of such metals followed by chemical analysis would show what effect they have upon the water. It may be noted that the water supply of most laboratories is pumped with pumps which are lined with brass and copper and is then carried for long distances through iron pipe, galvanized and ungalvanized, so that there is little use of making the last 60 feet of pipe of some special metal except where heat is applied.

TABLE 4

Showing the effect of the apparatus upon water. Analysis: solids in parts per million by Mariner and Hoskins and gases in cc. per liter

	HOT TAP	COLD TAP	APPARATUS
Oxygen.....		10.46	0.74
Nitrogen.....		18.45	3.33
Carbon dioxide.....		2.50	0.73
Half-bound carbon dioxide.....	31.85	32.5	31.34
Free ammonia.....	0.00	0.00	0.00
Albuminoid ammonia.....	0.04	0.04	0.05
Nitrates.....	1.70	0.70	0.60
Nitrites.....	0.00	0.00	0.00
Chlorine.....	12.00	10.00	12.00
Zinc, copper, aluminum and tin.....	0.00	0.00	0.00
Iron.....	0.15	0.06	0.11
Lime (CaO).....	13.60	48.40	13.60
Magnesia (MgO).....	16.57	18.82	20.01 ¹
Lead.....	0.02	0.01	0.01
Sulphuric acid (SO ₃).....	0.03	0.04	0.071

¹ Increases shown are due to resolution of precipitated salts as shown by repetition of analysis of the water and analysis of scale from the boilers by M. M. Wells. The magnesium in the boiled water probably varied from time to time and the statement that it was reduced (Shelford and Allee '12) is probably incorrect for most of the experiments.

The analysis shows that the differences between the mineral content of tap water and the water that passed through the apparatus are very slight. No traces of the various metals used in the construction of the apparatus appear in the water. There is a loss of 4.8 parts per million of calcium. Iron, magnesium and sulphuric acid were slightly increased. The absence of any traces of the metals contained in the apparatus is largely due to the fact that they quickly became covered with sediment and scale and the water did not actually come in contact with them. The boilers are of aluminum which also becomes covered over with aluminum oxide which is insoluble in water and only slightly soluble in acids. Aluminum is probably the best metal from which to make apparatus for biological purposes, principally for this reason.

The Chicago tap water usually contains quantities of algae, some rotifers, Protozoa and Entomostraca, the last usually dead. The boiling process kills and cooks any plankton in the water so that plankton-feeding fish might be able to secure food from the tap water but not from the boiled. This, however, is probably of no importance in the cases of the fish used in these experiments (see p. 220).

(2) *Introduction of gases.* Gases are introduced from tanks between the upper and lower coolers and by means of the gas introducer (fig. 1 A). The tank of gas is attached to the introducer by means of a rubber hose. The gas enters the chamber (*ch*) and passes through a number of the small openings. Large quantities of oxygen, carbon dioxide, or other soluble gases can thus be added. If it is desired to add a soluble gas directly to the tap water, valve *G* is opened and valve *F* is closed, thus allowing the water to flow directly into the delivery tank. It is necessary, when untreated tap water is used, to allow the gas to enter only in such quantities as will go into solution in the cooler coil, for if it comes off in bubbles other gases are also removed. When gases are to be added to water that has been boiled, but little gas is removed by any bubbles which may pass through, and here it is sometimes desirable to allow an excess to escape as bubbles by inserting a tee with the stem projecting upward between the rubber hose and the withdrawal cock.

Gas escapes in bubbles through the tee and bubbles are not introduced into the apparatus, where they interfere with the experiment.

III. MATERIAL

The material used in these experiments consisted of the following: Young of the common sucker (*Catostomus commersonii* Lac., 8-11 cm. long, adult length 45 cm.); adults and young of the golden shiner (*Abramis crysoleucas* Mit., 6-15 cm. long., adult length 15-20 cm.); a few adults and many young of the common shiner (*Notropis cornutus* Mit., 5-9 cm. long, adult length 12-20 cm.); young of the river chub (*Hybopsis kentuckiensis* Raf., 7-10 cm. long, adult length 15-20 cm.); young of the black bull-head (*Ameiurus melas* Raf., 13-15 cm. long, adult length 30 cm. or more); adults of the mud minnow (*Umbra limi* Kirt., adult length 10-13 cm.); young of the rock bass (*Ambloplites rupestris* Raf., 4-10 cm. long, adult length 20-35 cm.); young and adults of the blue spotted sunfish (*Lepomis cyanellus* Raf., 4-6 cm. long, adult length 10-18 cm.); young of the small-mouthed black bass (*Micropterus dolomieu* Lac., 8-9 cm. long, adult length 30-38 cm.); and adults of the rainbow darter (*Etheostoma coeruleum* Stor., adult length 5 cm.). The stock also included one or two adults of *Ameiurus nebulosis* LeSur., *Schilbeodes exilis* Nel., *Boleosoma nigrum* Raf., *Hadropterus aspro* C. and J., and one or two young of *Campostoma anomalum* Raf. and *Notropis atherinoides* Raf. On some occasions noted in the text, one or two of these were included in experiments with other closely related species. Others were used for preliminary experiments which are barely mentioned.

1. STOCK OF FISH

The *Ambloplites*, *Hybopsis*, *Micropterus*, *Notropis*, *Etheostoma* and *Schilbeodes* used in these experiments, were collected from Hickory Creek, New Lenox, Illinois, October 30, 1911. The *Boleosoma*, *Lepomis*, and *Catostomus* were taken at Flossmoor, Illinois, from Butterfield Creek, November 13. One or

² In the body of the paper the fishes are sometimes referred to by the generic names only, when it is to be understood that the species listed above are meant.

The analysis shows that the differences between the mineral content of tap water and the water that passed through the apparatus are very slight. No traces of the various metals used in the construction of the apparatus appear in the water. There is a loss of 4.8 parts per million of calcium. Iron, magnesium and sulphuric acid were slightly increased. The absence of any traces of the metals contained in the apparatus is largely due to the fact that they quickly became covered with sediment and scale and the water did not actually come in contact with them. The boilers are of aluminum which also becomes covered over with aluminum oxide which is insoluble in water and only slightly soluble in acids. Aluminum is probably the best metal from which to make apparatus for biological purposes, principally for this reason.

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Gas escapes in bubbles through the tee and bubbles are not introduced into the apparatus, where they interfere with the experiment.

III. MATERIAL

The material used in these experiments consisted of the following: Young of the common sucker (*Catostomus commersonii* Lac., 8-11 cm. long, adult length 45 cm.); adults and young of the golden shiner (*Abramis crysoleucas* Mit., 6-15 cm. long., adult length 15-20 cm.); a few adults and many young of the common shiner (*Notropis cornutus* Mit., 5-9 cm. long, adult length 12-20 cm.); young of the river chub (*Hybopsis kentuckiensis* Raf., 7-10 cm. long, adult length 15-20 cm.); young of the black bull-head (*Ameiurus melas* Raf., 13-15 cm. long, adult length 30 cm. or more); adults of the mud minnow (*Umbra limi* Kirt., adult length 10-13 cm.); young of the rock bass (*Ambloplites rupestris* Raf., 4-10 cm. long, adult length 20-35 cm.); young and adults of the blue spotted sunfish (*Lepomis cyanellus* Raf., 4-6 cm. long, adult length 10-18 cm.); young of the small-mouthed black bass (*Micropterus dolomieu* Lac., 8-9 cm. long, adult length 30-38 cm.); and adults of the rainbow darter (*Etheostoma coeruleum* Stor., adult length 5 cm.). The stock also included one or two adults of *Ameiurus nebulosis* LeSur., *Schilbeodes exilis* Nel., *Boleosoma nigrum* Raf., *Hadropterus aspro* C. and J., and one or two young of *Campostoma anomalum* Raf. and *Notropis atherinoides* Raf. On some occasions noted in the text, one or two of these were included in experiments with other closely related species. Others were used for preliminary experiments which are barely mentioned.

1. STOCK OF FISH

The *Ambloplites*, *Hybopsis*, *Micropterus*, *Notropis*, *Etheostoma* and *Schilbeodes* used in these experiments, were collected from Hickory Creek, New Lennox, Illinois, October 30, 1911. The *Boleosoma*, *Lepomis*, and *Catostomus* were taken at Floosmoor, Illinois, from Butterfield Creek, November 13. One or

² In the body of the paper the fishes are sometimes referred to by the generic names only, when it is to be understood that the species listed above are meant.

two *Lepomis* taken from the Fox River at Cary, Illinois, October 21, were included. The *Ameiurus*, *Abramis*, and *Umbra* came from a pond at Pine, Indiana, November 11. This stock was divided and one part was put in a standing-water aquarium and supplied with boiled water from time to time. This part of the stock will be referred to as the 'low oxygen stock.' All other fish were kept in aquaria supplied with running water from the tap. During the period of experimentation, the fish kept in good condition, with low mortality.

Most of the deaths were due to fungus attacking slight injuries or lesions, due to gas bubbles or handling. The fish fed largely upon small minnows which were present in the aquaria in large numbers; also upon fish foods and pieces of fresh water mussel. After December 1 the stock of fish was put into a large basement tank, supplied by the overflow from a large artificial pond on the campus. This water contained plankton and occasional invertebrates. The fish were kept all winter and noticeable mortality did not begin until April.

2. BEHAVIOR AND PHYSIOLOGY OF FISHES

Fishes usually remain active all winter (Abbott '75) apparently carrying on their regular activities as in warmer weather. The temperature of streams and of larger bodies of water probably does not fall below 4 to 6°C. before the end of December. Our experiments were performed in November and December, only a few being conducted in January. The behavior of fishes in autumn is not modified by the breeding activities and fishes may be brought to the laboratory in very large numbers without mortality and kept alive.

In mode of locomotion the fishes studied fall into two main classes. The first class comprises those that rest on the bottom much of the time, swimming by darts; this type includes *Boleosoma*, *Etheostoma*, and young *Catostomus*, the latter being somewhat more like the other fishes. The second class is made up of fishes that swim at a uniform rate, starting slowly. *Umbra* *Hypobysis*, and *Ameiurus* often rest upon the bottom; the other species do so rarely.

The fishes studied represent all degrees of gregariousness. The *Abramis*, *Hybopsis*, and *Notropis* are strongly gregarious. While they tend to follow any small fish, the most compact schools are made up of fishes of about the same size. Fishes larger or smaller than the majority are most likely to stray. Very compact schools of different species may be maintained if the fishes are about the same size. *Lepomis*, *Ambloplites* and *Microp-terus* are only slightly gregarious in captivity, *Lepomis* least of all. The *Umbra* and *Ameiurus* are least gregarious of the swimming fishes, two fishes rarely moving together. Of the darting and resting fishes, none are more than slightly gregarious.

IV. THE PHYSIOLOGICAL EFFECT OF GASES UPON FISHES

The physiological effects of gases upon fishes have been but little studied experimentally. Nothing has been done upon the species of fish which we used in the gas gradient experiments. While not a part of our main problem, we considered a knowledge of the effects of gases upon the species studied of importance and accordingly conducted some preliminary experiments. Some typical results of these are included here.

1. EFFECT OF A GREAT EXCESS OF NITROGEN AND OXYGEN

As has already been stated, the laboratory water supply contains an excess of gas at all times, this being especially true in the winter and spring. Fish kept in water which contains a large quantity of gas, usually develop gas bubble disease. Bubbles of gas, consisting largely of nitrogen (Marsh and Gorman '05) collect in the fins, beneath the skin of the head, behind the eyes, thus producing 'pop eye,' and in the circulatory system, especially in the heart, where they interfere with the circulation so as to cause death. Hitherto, the disease has been noticed especially in marine fishes.

Gas bubble disease developed in the stock of fishes during the progress of the experiments. The excess gas in the aquarium water ranged from 1 to 2 cc. per liter of both nitrogen and oxygen. Gas bubbles developed in the fins of *Ambloplites*, *Hybopsis*,

Notropis, Lepomis, Umbra and Ameiurus. No bubbles developed in Abranlis but they were experimentally produced on several occasions. A typical experiment consisted of raising the temperature of the water from 8 to 17°C. without loss of gas, and allowing it to flow into an aquarium. This gave a large excess of gas. Nine out of seventeen fish developed the disease in nine hours; four of these did not recover. The fishes of a standing water control at the same temperature, showed no signs of the disease. The cure of gas bubble disease was accomplished by bubbling gases through water in which diseased fish had been placed. Two Hybopsis and one each of Notropis, Catostomus and Ambloplites were cured of the disease in eighteen hours, by the bubbling of oxygen through a tall jar which contained the fishes. Two Umbra with large bubbles in their fins were placed in water through which nitrogen had been bubbled for thirty hours and the bubbling continued. Both fish were entirely cured in twenty-two hours. Table 3, experiment 3, shows that, making a very liberal allowance for exposure to the atmosphere, the nitrogen was probably increased 2 cc. per liter; (compare table 2). On another occasion, two Hybopsis which were badly affected, were cured in twenty-four hours in the same manner (table 3, experiment 4). Two Ambloplites and four Abramis were kept in water through which nitrogen was bubbled, for twenty-three hours, and neither showed signs of gas bubbles upon dissection, though one died of asphyxia due to the low oxygen content, 0.5 cc. per liter (table 3, experiment 5).

We find in these experiments *no suggestion that fish develop the disease as a result of a simple increase of gas when one gas is displaced by another, under one atmosphere of pressure*, but rather that the disease appears only where the gases are so much in excess that bubbles collect on any rough or warm object in the water. This excess may be due to a rise in the temperature of the water or a decrease in pressure, or both. It is probably essentially a laboratory disease (Birge and Juday '11, p. 134).

2. EFFECT OF A DEFICIENCY OF OXYGEN

Duncan and Hoppe. Seyler ('95, p. 165) found that the European cyprinid (*Tinca vulgaris*) is not affected by a prolonged exposure to oxygen reduced to 3 to 4 cc. per liter, but that when the oxygen supply is reduced below 1 cc. per liter they come to the surface and breathe heavily, violence of respiration increasing as the oxygen decreases. They were able to keep these fish alive, in a total absence of oxygen, for twenty-four hours. Trout were strongly affected by 1.7 to 0.8 cc. per liter in two or three hours. Reuss ('10) found that an increase of oxygen decreased breathing frequency. For further experiments and observations concerning the relation of fishes to gases, see Knauthe ('98, p. 785, '07, p. 148); Konig ('99, p. 32) and Marsh ('07, p. 346).

Several experiments were performed to determine the oxygen minimum for the different species. Table 5 represents our experience in this matter, together with current preferences which may roughly represent oxygen content of the natural environment.

The different rates of flow represent the following conditions: Swift water is high oxygen content; the category 'variable' may be construed as representing a condition in which stagnation occurs at times and accordingly represents conditions which

TABLE 5

Showing the relative time of succumbing to low oxygen content; and the current preferences of the same species after Forbes and Richardson ('08)

	LENGTH IN CM.	TURNING TIME ¹ IN MIN.	CURRENT PREFERENCES		
			Sluggish to swift	Sluggish to stagnant	Variable
Micropterus.....		20	55	18	27
Ambloplites.....	12	320			
Ambloplites, average.....	6	340	55	15	30
Hybopsis.....	10	355	53	24	23
Ambloplites.....	4	360			
Notropis.....	10	376	45	36	19
Abramis.....	10	400	32	57	11
Ameiurus.....		1080	37	53	10

¹The 'turning time' is the time before the fishes turned ventral side up

would be as detrimental to fish in the long run as a more constant low oxygen content. In our experience with low oxygen content, the smaller *Amploplites* are more hardy and the small *Abramis* are more sensitive than the adults. The data at hand suggest that there is some relation between habitat preference and the amount of oxygen necessary to maintain life.

3. THE EFFECT OF CARBON DIOXIDE AND AMMONIA

Many physiologists hold that carbon dioxide is more important than oxygen as a stimulant for respiratory action. In general its action is that of a narcotic, stimulating in small quantities, intoxicating in larger quantities, and producing death when taken in very large quantities. Similar behavior results have often been obtained with carbon dioxide and with acids. Both acids (Marsh '10, p. 896) and carbon dioxide are fatal to fishes when present in quantity. Reuss ('10, p. 555) worked with the effect of varying amounts of carbon dioxide upon the rainbow trout and found its general effect to be entirely similar to that with higher vertebrates. Up to about 15 cc. per liter, carbon dioxide acted as a stimulant to respiratory movements. Beyond this breathing did not become stronger. Staggering occurred with a concentration of from 25 to 41 cc. per liter and a total loss of equilibrium at from 44 to 53.5 cc. per liter. Weigelt ('85, p. 82) working with carp and trout, found that 35 to 37.5 cc. of carbon dioxide per liter had no effect but that 50 cc. per liter was sometimes harmful, while 100 cc. per liter was toxic at all times. He later ('03) reported 5 cc. toxic to *Tinca*. See also Knauthe ('07, p. 125).

A series of experiments including most of the species at hand confirmed the results of these workers with carbon dioxide. An *Ameiurus* was narcotized in 163 cc. of carbon dioxide per liter with oxygen at 1 cc. per liter. In this case the fish was placed in tap water and the amount of carbon dioxide present was gradually increased until anaesthesia was produced. Individuals of nearly all the species at hand were dropped into the water with the gas content as just given. They were all greatly stimulated at first, but lost correlation of movements in a few moments and died in ten to fifteen minutes.

Weigelt worked with ammonia (NH_3) and reports that 10 to 17 mg. of ammonia per liter had no effect on small fish and even 30 mg. per liter did not affect large ones, but smaller ones were affected by less than this amount. Fourteen milligrams are not uncommon in sewage, while 43 mg. have been reported.

V. REACTION OF FISH TO GASES IN SOLUTION

1. METHOD OF EXPERIMENTATION

It is impossible to study the reaction of fishes to gases without first establishing a gas gradient. Long boxes 120 cm. by 14 cm. by 20.5 cm. with screen partitions 5 cm. from the ends were constructed (figs. 2 and 3). The drain *D* placed at the center near the top consisted of a tube with screen bottom, opening outside. Water was allowed to flow in at both ends at the same rate (usually 600 cc. per minute) through tees perforated so as to distribute the flow across the tank (fig. 3). The two currents, too slight to interfere with the behavior of the fishes, met at the center of the box. The temperature and flow of the water into the ends must be the same if the gradient is to be perfect.³

Water which has been treated in the gas control apparatus, was introduced at the end *A* (fig. 3) so that a gradient was established in tank *AB*, while the control tank *BB* was alike at both ends. The oxygen content of the water in a typical boiled water gradient is shown in figure 2. The water coming from the tee

³ Tap water was introduced from $\frac{1}{8}$ -inch cocks into the tee (*T*) introducers at the end of the tanks marked (*B*, fig. 3) and at the same rate into end *A* from the gas control apparatus. The flow of water was regulated by shoving the rubber tubing connecting the tee introducer with the hose-end, onto the hose-ends as far as possible and ligaturing them securely with copper wire. Each rubber tube was wrapped with bicycle tape for a distance of about 3 cm. on each side of the termination of the metal hose-end. Another ligature was usually applied over the tape. A screw pinchcock was then placed over the wrapped portion of each tube. With the metal valve wide open each pinchcock was screwed down until the desired flow was secured; by measuring twenty seconds flow in a graduate the rate of flow was determined. The flow does not have to be adjusted more than once or twice a day and is almost constant for several hours. A metal valve is set for a given flow with much difficulty and the flow soon falls off because of the accumulation of sediment in the valve.

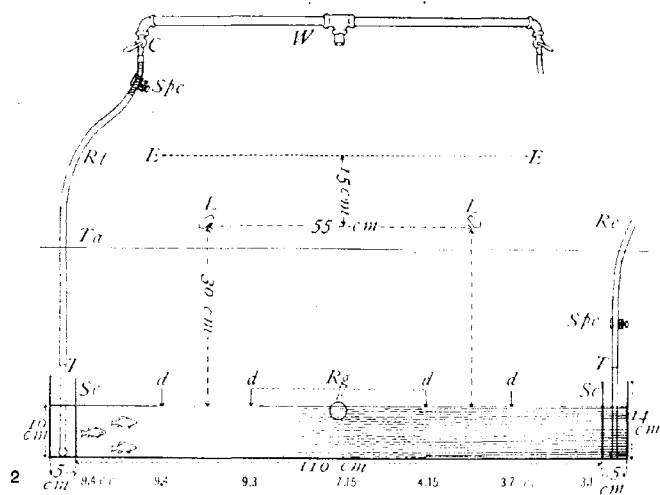
contained less than 1 cc. per liter but was increased by exposure to the atmosphere in the box. Samples taken from the boiled water end of the experimental tank contained more oxygen than was normally present at this end, due to the unavoidable disturbance of the gradient while the sample was collected. The experimental box was divided into three parts. Each end third was filled with water flowing in at that end, while the middle was a gradient between the two.

The complete apparatus used in the experiment is shown in figure 3. Two tanks as described above and shown in the figure were placed side by side in the bottom of an aquarium (in the absence of a suitable water table). The aquarium was enclosed beneath a black hood with side curtains as shown in figure 3. These side curtains hung loosely so that the observer could see into the hood and into the experimental tanks from either side.

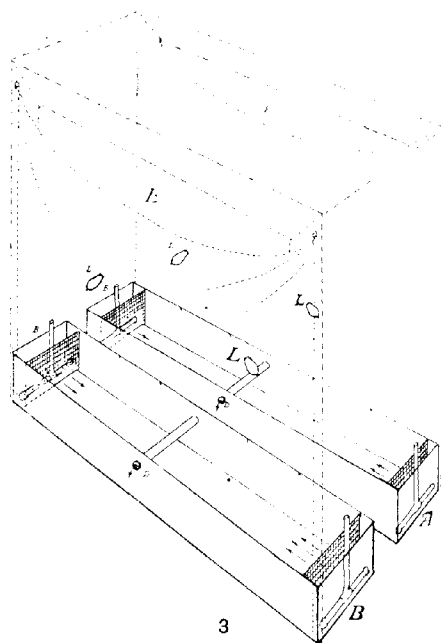
Fishes were placed in each of two dishes which were set above the experimental and control tanks respectively. Sheets of trial balance paper were prepared by recording kind, size, number and previous history of the fishes used, together with temperature, gas content of the water, et cetera. Certain vertical rulings on the trial balance paper were taken to represent the ends, center and thirds of the tanks. Usually the right-hand side of the paper was used for the main record. Vertical distance was used roughly to represent time.

Fig. 2 Shows the arrangement of the experimental tank in optical section, with distances and dimensions. *WS.* tap water supply; *C*, hose end cock; *Rt*, rubber tubing for tap water; *Rc*, rubber tubing leading to the apparatus; *Sp*, screw pinch cock over the tapped end of the hose; *EE*, level of the observer's eye, 45 cm. above the surface of the water; *L*, the 4-candle-power lights, 30 cm. above the surface of the water and above the center of the respective halves of the tank (the two lights being the only source of illumination) and between the observer and the fish; *Ta*, top of aquarium wall; *T*, is the tee introducer; *Sc*, the screen partition; *D*, drain; *Rg*, region of gradient; *d*, division points marked on the tank and corresponding to the red rulings of the trial balance paper. The series of figures, 3.1 to 9.4 cc., show the amount of oxygen present in collections taken from the gradient immediately above the location of the figures.

Fig. 3 Sketch of the experimental tanks and hood in position, with the arrows indicating the direction of the flow of water. The position of the lamps (*L*) and of the observer (*E*) is indicated. The tank lettered (*A*) at the right hand is the one usually used as the experiment in which case conditions are identical in the ends marked (*B*). Other lettering as in figure 2.



2



3

a. Reaction to carbon dioxide

The reactions of fishes to carbon dioxide in water are shown in table 6, with illustrative graphs in chart 1. The experiments fall into the following three classes: (a) Those with a difference of 5 to 10 cc. between the two ends; in these experiments the principal gradient was in the two center divisions as shown for oxygen in figure 2, page 227. (b) Those with a difference of about 19 cc.; in taking the readings of these, four central divisions were regarded as constituting the principal gradient. (c)

TABLE 6

Showing the reactions of fishes to a gradient of carbon dioxide in tap water. The experiments bearing numbers only, were performed with fishes taken directly from the stock aquaria, while the experiments bearing numbers and the letter A were performed with the same fishes, control and experimental individuals being interchanged. The control fishes of the first period of observation, after having become accustomed to going back and forth in the uniform tank were exposed to the gradient, while fishes accustomed to meeting the gradient were put into a tank where no gradient existed. For description of the apparatus, et cetera, see pp. 225-229; corresponding ends of the controls are designated by the same letters. H indicates high carbon dioxide and L low carbon dioxide.

EXPT. NO.	SPECIES	NO.	CO ₂ IN CC. PER LITER		CROSS-ED CENTER	TOTAL TIME IN MIN.	PER CENT OF TIME IN HALVES OF TANK				TURNED BACK IN GRADIENT FROM HIGH (H) OR LOW (L)				TEMP. DEG. C.					
			L	H			Expt.	Control	Expt.		Control		Expt.			Control				
									H	L	H	L	H	L		H	L			
85	Abramis.....	3	2	7	58	23	15	34	66	54	46	8	6	0	0	4				
87	Ambloplites.....	2	2	7	30	11	15	40	60	80	20	8	3	0	2	4				
87 X	Micropterus.....	1	2	7	13	6	15	46	54	79	21	4	1	0	1	4				
86 X	Hybopsis.....	1	2	7-13	12		9.5	15	85			3	0			4				
86	No. ropts.....	2	2	7-13	19	3	9.5	26	74	58	42	7	0	0	0	4				
46	Hybopsis.....	4	2.5	21	48	22	30	24	76	45	55	8	0	2	3	6				
46 A	Hybopsis.....	4	2.5	21	37	16	30	18	82	35	65	6	0	1	0	6				
44	Ambloplites.....	3	7	72	23	13	30	18	82	57	43	1	0	0	1	6				
45	Abramis.....	3	7	72	71	65	30	13	87	84	16	19	0	0	1	6				
45 A	Abramis.....	3	7	72	28	52	30	8	92	53	47	6	1	11	5	6				
47	Lepomis.....	4	12	80	69	101	30	42	58	50	50	16	2	4	6	6				
47 A	Lepomis.....	4	12	80	46	58	30	8	92	70	30	19	1	1	3	0				
48	Ameiurus.....	2	12	80	34	46	30	12	88	45	55	13	0	6	4	6				
48 A	Ameiurus.....	2	12	80	15	24	30	10	90	32	68	9	0	12	5	5				
49	Umbra.....	2	12	80	14	23	30	5	95	40	60	10	2	4	2	5				
49 A	Umbra.....	2	12	80	22	37	30	16	84	38	62	13	0	16	12	5				
52	Catostomus.....	3	15	80	100	4	30	25	75	2	98	11	0	0	4	5				
52 A	Catostomus.....	3	15	80	39	85	30	23	77	40	60	17	4	2	5	5				
53	Notropis.....	3	6	71	46	133	20	13	87	48	52	41	7	5	4	5				
53 A	Notropis.....	3	6	71	64	44	20	20	80	40	60	41	2	0	2	5				
Totals.....							54	150	1081	788	760	494	116	1584	871	929	200	29	64	60
Average.....								7.5	51	39	38	24	21	79	48	52	13	2	3	3

Those with a difference of about 65 cc. between the two ends, the whole tank being regarded as gradient because the carbon dioxide drifted across to the tap water end so as to raise the content from 5 to 13 cc. The amount of carbon dioxide was markedly less at the surface, due to loss into the atmosphere.

Occasionally the fishes, when put into the center, moved into the tap water end and came to rest without encountering the high carbon dioxide. In such cases they were driven to the center with the hand or were roused by jarring the tank and a corresponding number of drivings or tappings was effected in the control. The reactions of the fishes to the carbon dioxide gradient fall into the following three main classes: (a) They entered the carbon dioxide water with little perceptible hesitation and gave some definite reaction while there. (b) They gave an avoiding reaction upon reaching the increased dioxide. (c) They reacted by rising to the surface.

The reactions of the first type were given by most of the swimming species usually at the beginning of the experiment in which carbon dioxide exceeded 20 cc. per liter. With the exception of the Abramis, the fishes gave a coughing reaction in which the mouth was thrown wide open and the jaws slightly protruded with a sudden jerk. The same reaction sometimes took place more slowly and may be characterized as a yawn. In all cases observed, the gill movements were increased. The second type of reaction usually occurred after the fishes had tried the highest carbon dioxide and had given one of the reactions just mentioned. The commonest of these reactions may be characterized as a testing or *backing-starting* reaction. The fish moved forward and acted as though it encountered a sheet-rubber wall which it carried forward for a short distance but which in turn through its elasticity, caused the fish to rebound for an equal distance, probably 2 to 10 mm. In other words, the fish suddenly stopped, backed a very short distance and immediately moved forward the same distance again, usually repeating several times. The fishes sometimes turned back after giving this reaction, and sometimes turned back without giving it, and without any other characteristic movement. The third type of reaction was sometimes given following the other two but was especially charac-

which was followed by resting in the low for two minutes. In experiment 53 *Notropis* showed similar alternations of invasions and avoidances which took place more rapidly because the fishes moved back and forth more rapidly. *Notropis* (experiment 86, chart 1) invaded the high concentration only once after the first minute. In the controls for all the experiments, the fishes moved back and forth with more or less regularity and on the average with little apparent preference for either end.

An inspection of table 6 and the graphs (pp. 230-233), shows that activity is much greater in the experiments where low concentrations were used, as indicated by the number of crossings of the center. The activity where high concentrations were used was variable. The percentage of time in the low half of the experiment is in all cases much greater than in the high. Time percentage and number of turnings back in the gradient portion are the best indications of reaction. The number of turnings back from the higher concentration was greatest in all cases. In the controls, the number of turnings was usually nearly equal.

The reactions of all the species of fish experimented upon are quite similar although they belong to several taxonomic groups. The data in table 6, if averaged, show an almost equal number of crossings of the center in the experiment and control, but there is a marked time preference for the low carbon dioxide half of the experimental tank and a nearly equal division of time between the two ends of the control. The average number of turnings back from the higher concentration is thirteen as opposed to one from the lower. The average turnings in the controls are three from each half.

Preliminary experiments were tried with *Schilbeodes*, *Etheostoma*, *Boleosoma*, and *Hadropterus*. While the experiments were not carried far enough to give results of definite value they suggest that these swift water fishes which probably encounter very little carbon dioxide, may react less definitely to it than the fishes which live more often in the presence of carbon dioxide.

Table 7 shows the reactions of *Abramis* from the low oxygen stock and *Hybopsis* to a carbon dioxide gradient in boiled water

with oxygen 2.4 cc. per liter. The reactions were more marked, as shown by the graph (chart 1, experiment 55, p. 233), but when compared with other experiments, the depressing effects of the low oxygen and carbon dioxide are suggested. The fishes showed greater disturbance in these experiments than in the presence of more oxygen.

TABLE 7

Showing the reactions of fishes to a carbon dioxide gradient in boiled water. Oxygen content of the water was from 1 to 2.4 cc. per liter. Other data as in table 6.

EXPT. NO.	SPECIES	NO.	CO ₂ IN CC. PER LITER		CROSS- ED CEN- TER	TOTAL TIME IN MIN.	PER CENT OF TIME IN HALVES OF TANK				TURNED BACK IN GRADIENT FROM HIGH (H) OR LOW (L)				TEMP. DEG. C.			
			L H				Expt. Control	Expt. Control		Expt. Control		Expt. Control						
			L	H				II	L	II	L	II	L	H		L		
70	Hybopsis.....	3	2	5	50	0	8	110	10	2	98	54	46	9	0	3	6	7.5
70 A	Hybopsis.....	3	2	5	50	0	15	2	10	4	96	80	20	9	0	0	0	7.5
84	Abramis.....	3	2	5	24	0	36	87	27	12	88	29	71	75	4	41	4	8.5
84 A	Abramis.....	3	2	5	24	0	21	76	15	9	91	63	37	34	0	4	24	8.5
Totals.....			80		275	27		373	226	174	127	4	48	29				
Averages.....			20		69	7		93	56	44	32	1	12	7				

TABLE 8

Showing the reactions of fishes to a carbon dioxide gradient in experiments lasting forty minutes or more. The numbers from 1 to 6 refer to six equal longitudinal divisions of the tanks (indicated by the d's in fig. 2) when counted from the low (L) carbon dioxide end of the experimental tank and the corresponding end of the control tank. The numbers beneath them represent the percentage of total individuals (number of individuals times number of readings) recorded in each division at the time of the readings. Readings were taken every five minutes.

SPECIES	EXPT. NO.	NO. READ.	NO. FISH	CO ₂ IN CC. PER LITER		L—EXPERIMENT—II										CONTROL					
				NO. OF TANKS	PER LITER	Expt.										L					
						L	H	1	2	3	4	5	6	1	2	3	4	5	6		
Ambloplites.....	63	15	8	3	6	50	69	7	21	3	0	0	0	42	5	10	12	5	26		
Lepomis.....	64	12	5	3	6	50	63	22	15	0	0	0	0	23	16	3	11	11	36		
Abramis.....	65	8	12	16	6	50	88	12	0	0	0	0	0	34	21	12	5	16	12		
Average, per cent.....						73	14	12	1	0	0	0	33	14	8	9	11	25			

The results of several long experiments with readings every five minutes are shown in table 8. Here the fishes showed a

marked preference for the low carbon dioxide, which was maintained for more than an hour. They showed no tendency toward becoming acclimated to the carbon dioxide during this period.

b. Reactions to oxygen

In the experiments in which oxygen alone was varied, boiled water was used at both ends and oxygen added at one end. Titrations of collections from the ends of the tank showed a gradient of 4 to 10 cc. per liter, but as has already been stated, these collections tended to disturb the gradient so that exact differences could not be determined. The water as it left the deaërating machine contained about 1 cc. of oxygen per liter and this probably gave a minimum gradient of 5 cc. per liter at the bottom of the experimental tank. The reactions of the fishes

TABLE 9

Showing the reactions of fishes to an oxygen gradient in boiled water. Numbers and abbreviations as in table 6, p. 230. Experiment 72x was run with different fishes from Experiment 72 because the fishes in 72 were not accustomed to seeing the experimenters and the results were interfered with by fright. Abbreviations as in Table 6.

EXPT. NO.	SPECIES	NO.	O ₂ IN CC. PER LITER		CROSS-ED GEN-TER		TOTAL TIME IN MIN.	PER CENT OF TIME IN HALVES OF TANK				TURNED BACK IN GRADIENT FROM HIGH (H) AND LOW (L)				TEMP. DEG. C.
			L	H	Expt.	Control		Expt.		Control		Expt.		Control		
								H	L	H	L	H	L	H	L	
75	Lepomis.....	3	1.2	11.7	59	22	20	58	42	44	56	11	4	2	1	{Exp.9 Con.7
72 X	Notropis.....	3	3.7	7.9	67	33	20	75	25	66	34	12	43	2	2	
72	Notropis.....	3	3.7	7.9	68	9	20	46	54	48	52	6	12	1	1	8
73	Hybopis.....	3	3.4	7.9	38	49	20	73	27	42	58	3	5	4	2	9
73 A	Hybopis.....	3	3.7	7.9	80	125	20	56	44	54	46	4	13	6	0	9
74	Catostomus.....	3	3.7	7.9	29	163	20	56	44	53	47	1	7	7	4	9
74 A	Catostomus.....	3	3.7	7.9	135	3	20	48	52	41	59	5	30	0	0	9
76	Abramis.....	3	1.2	11.7	35	34	20	27	73	46	54	2	0	0	0	9
69	Ambloplites.....	3	1.2	12.0	6	15	20	53	47	21	79	1	1	0	0	8
69 A	Ambloplites.....	3	1.2	12.0	9	19	20	71	29	46	54	0	2	0	1	8
Average.....			2.7	9.5	52.6	47	20	56	44	46	54	4.5	12	2	1	

¹ One fish lay in the low oxygen over four minutes; cause unknown; control often resting.

² No evidence of a reaction.

³ Fishes swam at the surface of the low oxygen end for seven minutes and thus were giving a reaction to the vertical, but not to the horizontal gradient.

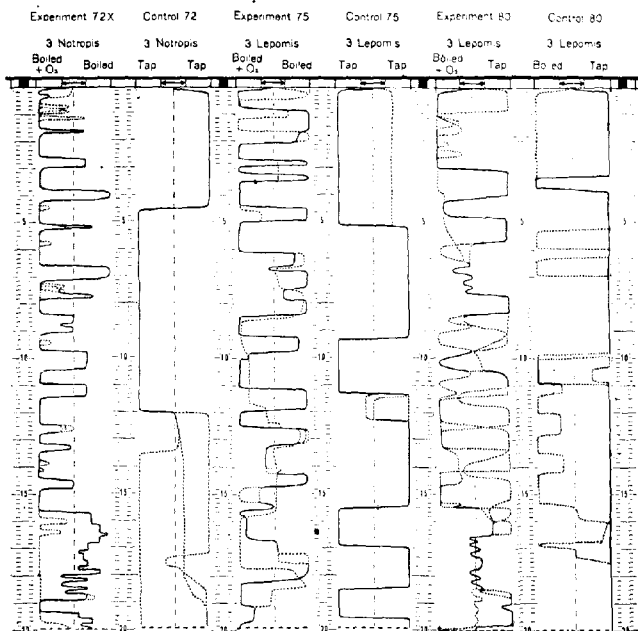


Chart 2 Showing the reactions of fishes to an oxygen gradient in boiled water and to the effect of boiling. For oxygen concentration, see table 9; for experiment 80, table 15. For a description of the method of charting, et cetera, see chart 1, p. 233.

(table 9) were in general very indefinite, but the activity was greater in the experiment, due to the stimulating effect of the change in character of the water in passing from one end of the tank to the other. Time preference was not strongly in favor of the high oxygen end except in experiment 72x (chart 2). In 72 fright appeared to enter into the behavior and there was a time preference for the low oxygen end. In all cases, turnings in the gradient were more numerous from the low than the high, though the differences from the control were not so great as in the case of carbon dioxide. Chart 2, experiment 72x,

shows the graph of the reaction of *Notropis*, which was the most decided response given. It will be noted that *Notropis* turned back from the low concentration several times during the first fifteen minutes of the experiment but became very indefinite during the last five minutes. A typical reaction of *Lepomis* is shown in chart 2, experiment 75, of *Hybopsis* in chart 4, experiment 73. The former was plainly indefinite in its reactions throughout, but turned back more often from the higher concentration. *Ambloplites* and *Abramis* did not turn back in the gradient and gave no definite reaction. For the other fishes, the number of turnings in the gradient indicates some reaction to the absence of oxygen.

TABLE 10

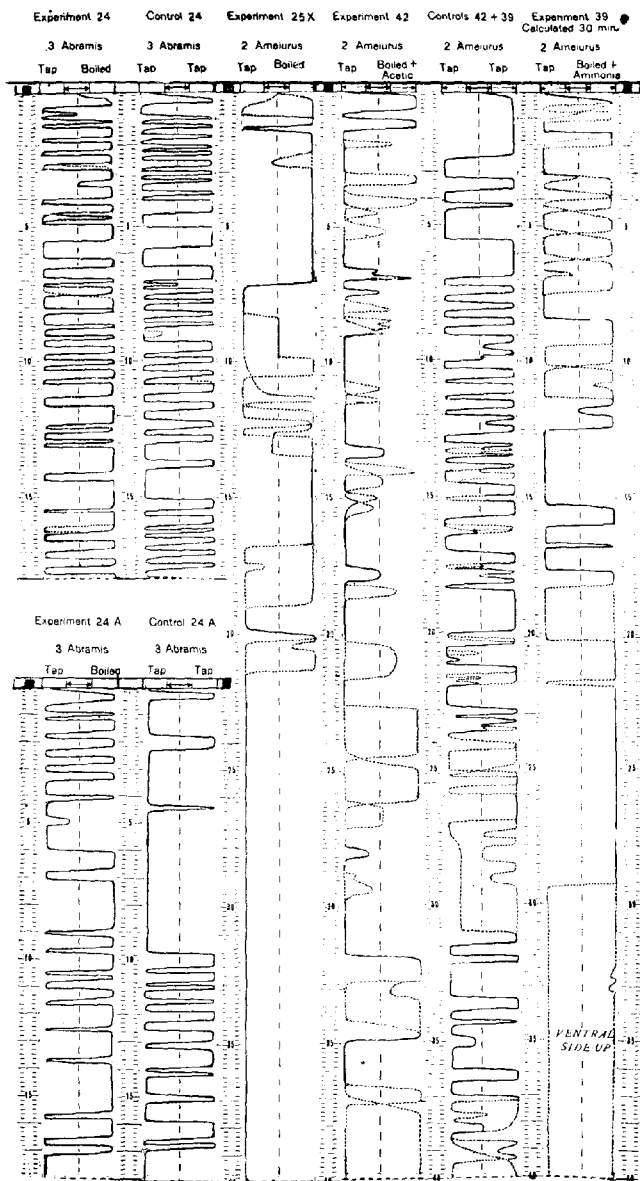
Showing the reactions of fishes to a nitrogen gradient. The gradient was established by running boiled water into both ends of the experimental tank and adding the high nitrogen atmosphere at one end and enough oxygen to balance the oxygen added with the nitrogen, at the other. Control in tap water. The difference in nitrogen secured was only 3 cc. per liter.

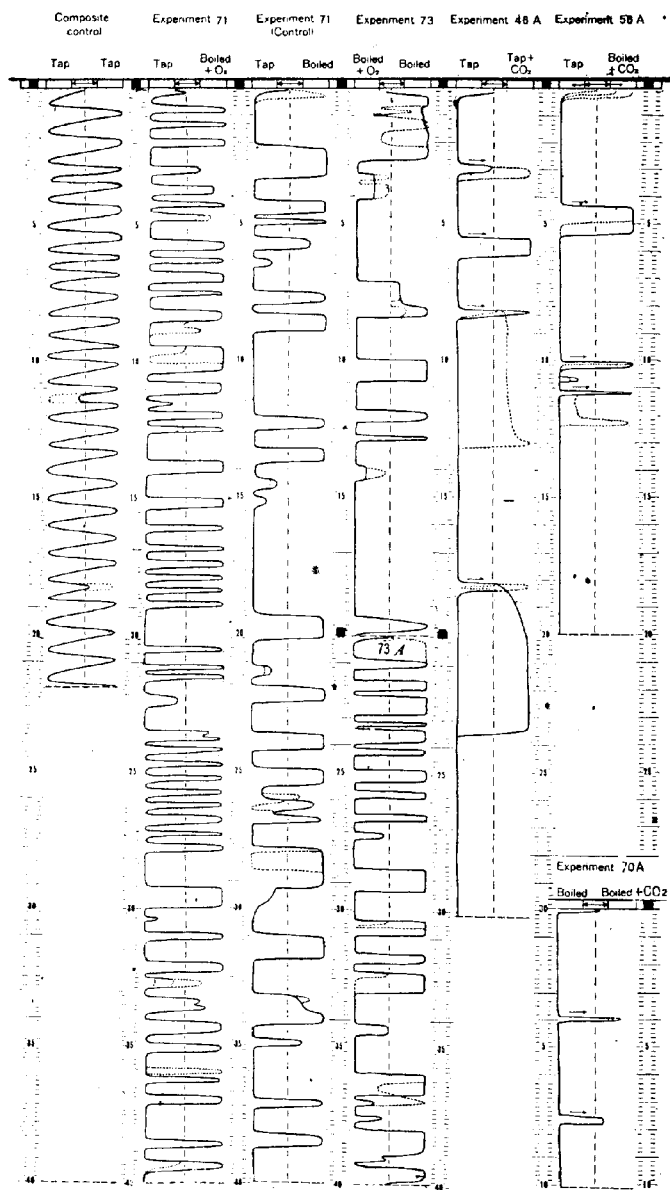
EXPT. NO.	SPECIES	O ₂ AND N ₂ IN CC.		CROSS- ED CEN- TER	TOTAL TIME IN MIN.	PER CENT OF TIME IN HALVES OF TANKS				TURNED BACK FROM HIGH (H) OR LOW (L)				TEMP.		
		O ₂				Expt.		Control		Expt.		Control				
		I ₂				N ₂		N ₂		H		L				
		I ₂				H		L		H		L				
		O ₂	O ₂	Expt.	Control											
		2	28.2	44												
66	<i>Hybopsis</i>	3	4	7	33	13	20	17	83	52	48	1	0	4	0	10
66 A	<i>Hybopsis</i>	3	4	7	12	69	20	31	69	32	68	0	1	0	3	10
67	<i>Notropis</i>	3	4	7	60	00	20	26	74	59	49	0	3	8	12	10

c. Reactions to nitrogen

We were unable to secure pure nitrogen and did not succeed in putting enough of this inert gas into solution under one atmosphere pressure to duplicate the high nitrogen content which is sometimes found in the deeper waters of lakes. Two preliminary

Chart 3 Showing the reactions of low oxygen *Abramis* to boiled water and of *Ameiurus* to boiled water with acetic acid or ammonia added. Ammonia was used in experiment 39; acetic acid in experiment 42. For detailed discussion, see pp. 247, 252 and tables 13, 17 and 18. For a description of the method of charting, see chart 1, p. 233.





experiments were run with the two most sensitive species *Hybopsis* and *Notropis*). The oxygen content of the water was raised by the addition of the atmosphere from the tank (nitrogen 92 parts; oxygen 8 parts) and a small quantity of oxygen was added at the other end. This made the difference in the oxygen content of the two ends only 0.16 cc. per liter with the most oxygen at the high nitrogen end. In the three experiments, there were no turnings but a time preference for the low nitrogen end. The graph of the reaction of *Hybopsis* gives no good evidence that the fish reacted to nitrogen but only a suggestion that they may react to a nitrogen gradient, and that they may select the low concentration. However the fishes may have been avoiding a slight oily odor which was detectable in the high nitrogen atmosphere. Because of this and the small difference in nitrogen that could be obtained and the difficulty of manipulation, the experiments were not carried further.

3. REACTIONS TO COMBINATION OF FACTORS

a. Boiled water

The effect of boiling the water in the apparatus is shown in table 4 (p. 217). The water lost most of its oxygen and much of its nitrogen, the nitrogen content being reduced from 18.45 to 3.33 cc. per liter. The free carbon dioxide was reduced from 2.5 to 0.7 cc. per liter; 1.2 cc. per liter of half-bound carbon dioxide was lost in addition to the changes in salt content already discussed. The fishes were then reacting to a difference in salts, carbon dioxide and nitrogen and oxygen. When the higher oxygen was chosen, the fishes of necessity selected the higher nitrogen and the higher carbon dioxide. We have clear evidence that the fishes selected the lower concentrations of carbon dioxide when the minimum was that of tap water but we have no evidence concerning the optimum amount of carbon dioxide for fish. It is therefore difficult to interpret the results of such

Chart 4 Showing the relative intensity of reaction to the various factors employed in experiments with *Hybopsis*. The control given is a graphic representation of the average of all the controls.

experiments as we are about to describe. The stock used was divided into two parts; one in tap water, and the other in water with less than 1 cc. of oxygen per liter.

1. *High oxygen stock.* Nearly all the fishes kept in tap water reacted with some distinctness to the boiled water, but considering only time preferences, the *Etheostoma* and the *Ambloplites* did not react to the gradient, since they showed a time preference for the boiled water in some experiments and for tap water in others. All the others gave a preference for the tap water, though in many cases it did not exceed that shown for one end of the control where the two ends were identical. For example, the control individuals of *Hybopsis* (experiment 10) spent 90 per cent of the time in one end of the tank, the experimental fishes only 76 per cent in the tap water of the experiment. Such cases are explainable when we consider the amount of activity as indicated by crossing the center in experiment and control. The control fishes in this case crossed the center only one-sixth as many times as the experimental fishes. This was characteristic and was probably due to the stimulating effect of encountering a change of water.

The fishes which reacted with greatest precision to the boiled water gradient were *Hybopsis* (chart 4, experiment 71), *Micropterus*, and *Notropis* (table 11). The reaction of *Lepomis* in experiment 13 serves as a typical case of the reactions to the boiled water. The fishes showed a time preference for the tap water and ten turnings back from the boiled against six from the tap. The turnings of *Lepomis* and *Hybopsis*, which react similarly, were not characterized by any striking movements of the mouth or opercles though in the experimental tank the fishes showed a disturbance due probably to lack of oxygen. That is, they gave characteristic risings to the surface and gulpings with emission of air bubbles, in the boiled water end, and some hesitation in crossing the center, not shown in the control. *Micropterus* is apparently one of the most sensitive of fishes. Our original stock consisted of only four specimens, three of which died in water containing less than 1 cc. of oxygen, while confined there in the second experiment attempted (see p. 245). In the one

experiment performed the experimental fishes tried the boiled water three times during the first eight minutes. During the last half of this period they showed some disturbance in excess of that shown in the control, by rising to the surface and opening the mouth. At the end of eight minutes the fishes showed their first tendency to turn back at the center. After trying the boiled water three times more, they began turning quite regularly. This was continued twenty-six minutes with trials of the boiled end being made every five minutes. The trials were accompanied by some of the typical avoiding reactions, especially by rising to the surface until the fins protruded.

The graph of the reaction of *Notropis* (experiment 2, table 11) showed less definite turnings than *Hybopsis* or *Lepomis* and

TABLE 11

Showing the reaction of fishes from the high oxygen stock to a boiled water gradient. The meaning of abbreviations, et cetera, is essentially as in table 6, p. 230. Controls in tap water. Gradient between tap and boiled.

EXPT. NO.	SPECIES	NO.	LENGTH IN CM.	O ₂ IN CC. PER LITER		CROSS-ED CENTER	TIME IN MIN.	PER CENT OF TIME IN HALVES FROM BOILED (B) (TAP, T, BOILED, OR TAP (T) IN GRADIENT B)								
								T				B				
								Expt.	Control	Expt.	Con.	Expt.	Con.	Expt.	Con.	
				H	L	Expt.	Control									
13	Lepomis.....	3	4-5	9.4	3.1	112	77	60	67	33	49	51	6	10	7	6
3	Ambloplites.....	3	5-7	8	1	113	91	60	73	27	73	27	5	13	1	5
9	Micropterus.....	2	8-9	9.4	3.1	43	29	40	64	36	85	15	4	8	0	0
21	Etheostoma.....	3	adult	9.4	3.1	77	59	40	55	45	38	62	1	2	7	4
21 A	Etheostoma.....	3	adult	9.4	3.1	33	57	40	41	59	64	26	6	3	4	2
12	Catostomus.....	3		9.4	3.1	106	3	30	64	36	28	72	7	11	0	0
4	Ameiurus.....	3	13-15	8	1	55	52	30	85	35	49	51	2	0	3	4
1	Abramis.....	3	6-10	8.1	0.5	27	37	60	80	20	41	59	0	0	3	3
1 A	Abramis.....	3	6-10	8.1	0.5	21		60	62	38			0	3		
11	Ambloplites.....	3	5-6	9.4	3.1	40	69	60	27	73	60	31	0	1	2	8
10	Hybopsis.....	3	7-9	9.4	3.1	270	46	40	76	24	10	90	3	78	2	3
2	Notropis ³	3		8.0	1.0	248	36	{ 60F. 30C }	69	31	14	56	0	73	0	0
Average.....									62	38	47	53	3	14	3	3

¹ No reaction to the gradient.

² The fishes first encountered the boiled water which depresses the general activity of *Ambloplites*, p. 245.

³ Two *N. atherinoides* were included in the experiment here by mistake, which may account for a more definite reaction than was shown in table 15, experiments 77, 77 A and 79, where no *atherinoides* occurred.

avoidance did not begin until the fishes had tried the boiled water about twenty-five times during the first twenty-five minutes. In the *Hybopsis* experiments the fishes began to turn back after about fifteen trials of the boiled water during the first ten minutes. The testing or backing-starting reaction was given when the boiled water was encountered and while the fishes were turning back. The experimental fishes began rising to the surface and taking the surface film for respiration at about the time the turnings at the center became frequent. In spite of the marked difference in reflexes, *et cetera* *Ameiurus* reacts similarly, as shown in experiment 4, table 11. The control fishes went back and forth quite symmetrically, turning at the center when going in either direction and showing no marked preference for either end. The experimental fishes reacted to boiled water by showing a time preference for the tap water, by vigorous opercular movements, and by gaping and rising to the surface. There were no turnings back from the boiled water and the two from the tap water are due to the fact that the fishes thus turning were swimming at the surface of the water and encountered the center drain.

The behavior of *Abramis* (experiments 1 and 1 B, table 11) was in some respects similar to that of *Notropis*. The control behavior was very similar, being simple symmetrical back-and-forth movements in both cases. The behavior of *Abramis* in the experiment was however different, in that the fishes rarely turned back more often from one-half than the other but established an apparent preference for one end and did not move out of it.

Ambloplites is the most peculiar of the swimming fishes which we have studied. In experiment 2 (table 11) the control graph would make a good experimental graph for most of the fishes. In this case the fishes acquired an apparent preference for the end of the control corresponding to the tap water of the experiment and turned back eight times from the half corresponding to the boiled water. In the case of the experimental fishes, on the other hand, two of the three moved into the boiled water when they were put into the center at the beginning of the experi-

ment. The third, which first moved into the tap water, soon joined the other two fishes and all remained in the boiled water for thirty-four minutes. Then all began moving back and forth showing a time preference for the tap water but without turnings or other characteristic activity. In experiment 3 (table 11), on the other hand, all the fishes moved to the tap water end of the tank at the beginning and remained four minutes except for a single excursion into the gradient. After this time they began to go into the boiled water but showed a marked time preference for the tap water end. In the box experiments (p. 230) and elsewhere, the boiled water decreased the activity of *Ambloplites* so that if for any reason the fishes came to rest in the boiled water for a time, their tendency to leave was decreased rather than increased, as is usually the case.

The darting fishes, *Catostomus* and *Etheostoma*, reacted to the boiled water and in fact to all differences in water in a somewhat different way from the swimming fishes. In the experiment the reactions of the fishes were most erratic. All of their movements were dartings and restings, accompanied by risings to the surface. *Catostomus* gave off bubbles of air and in two or three cases leaped out of the water. However, they finally worked out an apparent preference for the tap water.

Long experiments with five or ten minute readings were conducted with most of the species. Since some of the fishes, such as *Abramis*, often established an apparent preference for one end or the other without testing both, it was thought advisable to confine the fishes in the boiled water for a time to permit them to become affected by the boiled water before the readings began. While this later proved to be of little advantage and often undesirable, it was continued to make the series uniform. The results are shown in table 12, page 246.

Notropis which reacted definitely in the closely observed experiments (tables 11 and 15) here shows a decided preference for the boiled water. The fishes began rising to the surface—one of their very definite reactions to unsuitable water—and kept this up throughout the experiment. They tend to move about in circles near the same spot when giving this reaction and

while it is a definite reaction to the boiled water, the fact is not evident from the table alone. *Lepomis* were indefinite in their reactions, showing a slight preference for the boiled water in one case and for the tap in another. *Abramis* showed a clear preference for the tap water or tap water and gradient; *Ameiurus* showed a preference for the boiled water in the second two experiments. The *Ambloplites* showed the same reversal of preference as was shown in the earlier experiments. *Catostomus* showed a slight preference for the tap water while *Etheostoma* showed a preference for the boiled water, as in one of the experiments described above. When we note the decided preference for one end or the other in the controls, we are justified in concluding that on the whole, the reactions of the fishes to the boiled water during the longer periods was indefinite rather than clear-cut.

TABLE 12

Showing the reaction of the fishes from the high oxygen stock to a boiled water gradient in experiments lasting an hour or more. Since some of the fishes tended to rest in the tap water end of the experiment and not to come into contact with the boiled water, they were confined in the sixth of the tank nearest the boiled water end for thirty minutes or more. At the beginnings of the readings they were released and usually went back and forth in the tanks. For the distribution of the gradient in these experiments, see figure 2, p. 227. The boiled water at the point of inflow, contained less than 1 cc. of oxygen per liter. Nearer the surface the amount was probably larger. Controls in tap water; confined as experiments.

EXPT. NO.	SPECIES	TIME CONTINUED BOILED IN MIN.	EXPERIMENT			CONTROL			NO. FISH	TIME BTW. READ.	NO. READINGS
			Tap	Gradient	Boiled	Cor. tap	Cor. Gradient	Cor. boiled			
18	<i>Abramis</i>	35	69	7	24	8	8	84	7	5	14
18 A	<i>Abramis</i>	35	55	1	44	25	41	34	7	5	13
18 A	<i>Abramis</i>	35	41	52	7	38	37	25	7	5	13
19	<i>Lepomis</i>	55	51	9	40	21	25	54	5	5	12
19 A	<i>Lepomis</i>	30	14	42	44	32	29	39	5	5	19
16	<i>Ambloplites</i>	50	43	10	47	55	20	25	5	5	12
16 A	<i>Ambloplites</i>	30	83	5	11	39	0	41	5	5	13
14	<i>Catostomus</i>	4	52	28	30	53	7	40	7	¹⁰ ₁₅	28
15	<i>Catostomus</i>	39	31	10	26	25	21	51	6	2	41
17	<i>Ameiurus</i>	35	30	33	28	31	22	47	3	5	12
17 A	<i>Ameiurus</i>	36	7	0	93	28	61	11	3	5	12
17 B	<i>Ameiurus</i>	50	7	0	93	22	50	28	3	5	12
20	<i>Notropis</i>	35	2	87	11	38	15	47	5	5	18
20 A	<i>Notropis</i>	30	0	0	100	97	0	3	5	5	12
30	<i>Etheostoma</i>	32	21	1	78	41	31	28	3	5	13
30 A	<i>Etheostoma</i>		13	27	60	13	30	67	3	5	16

In the closely observed experiments, Abramis, Hybopsis and Notropis may be said to have reacted with considerable precision, either by rising to the surface or by spending more of the time in the high end or by turning back definitely from the center. In the long experiments, the amount of rising to the surface was not definitely noted in all cases although this reaction may dominate over all others. Ameiurus and Catostomus sometimes give this reaction so the preference of these fishes for the boiled water as shown in table 12 is open to some question.

2. *Low oxygen stock.* The low oxygen stock consisted of a number of Abramis, Ameiurus and Umbra. They were put in a small glass-sided aquarium and supplied with water from the boiling apparatus, which water was changed every few days and which always showed an oxygen content of less than 1 cc. per liter. The carbon dioxide at times was as high as 8 cc. per liter. Tables 13 and 14 give the results of experiments upon this stock

TABLE 13

Showing the reactions of fishes from the low oxygen stock to a boiled water gradient.

The data are arranged as in the preceding tables; compare with table 11. Controls in tap; gradient between tap (T) and boiled (B); corresponding ends of the control designated by the same letters. Tap water contained 8 cc. per liter. Boiled less than 1 cc. per liter.

EXPT. NO.	SPECIES	NO.	CROSS- ED CEN- TER	TIME IN MIN.	PER CENT OF TIME IN HALVES				TURNED BACK FROM TAP (T) OR BOILED (B)				TEMP. IN DEG. C.		
					Expt.		Control		Expt.		Control				
					T	B	T	B	T	B	T	B			
24	Abramis.....	3	330	438	40	49	50	5	55	45	4	9	1	6	17
24 A ¹	Abramis.....	3	106	87	20	44	56	79	21	1	6	0	0	0	17
25 ¹	Umbra.....	3	45		40	51	46				1	3			17
25 A	Umbra.....	3	68	106	40	59	41	52	48	6	7	0	1	1	17
22	Abramis.....	3	128	63	10	67	33	35	65	6	10	6	6	6	19
23	Abramis.....	3	425	360	30	41	59	50	50	19	12	4	6	6	12
7	Umbra.....	3	25	28	40	61	39	34	66	3	5	1	0	0	7
25 X	Ameiurus.....	2 ³	23	3	40	64	36	49	60	3	1	0	0	0	18
Average.....			143	155	12	55	45	49	51	5	2	2	3		

¹ Fish same as 24, left in tank one hour and forty minutes, and read again.

² Control reading incomplete.

³ Three fishes in control.

in low oxygen. *Abramis* was apparently affected by the continued exposure to low oxygen. In the high oxygen stock, there was a definite apparent time preference for the tap water while here in three of the four trials the fish gave an apparent time preference for the boiled water. In one experiment the turnings were more numerous from the tap than from the boiled water. When all the turnings are considered as of the same value as the time preference, positive reaction is markedly less than that shown by the high oxygen stock. The same thing is shown in the long experiments (table 14). This difference in reaction may

TABLE 14

Showing the reactions of fishes from the low oxygen stock, to a boiled water gradient. Data arranged as in the preceding tables. Compare with table 12, p. 246. These fishes were confined in the tap water and for 30 minutes or more. Controls in tap water.

EXPT. NO.	SPECIES	NO.	TEMP. DEG. C.	EXPERIMENT TAP, T; BOILED, B				CONTROL T CORRESPONDS TAP B CORRESPONDS BOILED				TIME BETWEEN READINGS
				T	Gradi- ent	B	No. read.	T	Cor. Grad.	B	No. read.	
28	<i>Abramis</i>	7	21	26	27	47	14	35	26	39	14	5
28 A	<i>Abramis</i>	7	19-21	19	2	79	14	43	14	43	14	5
26	<i>Umbr</i>	3	19	28	46	26	13	59	18	23	13	5
26 A	<i>Umbr</i>	3	19	41	13	46	13	72	7	21	13	5
27	<i>Ameiurus</i>	3	16	20	49	31	13	4	36	60	13	5
27 A	<i>Ameiurus</i>	3	16	13	49	38	13	8	69	23	13	5
5	<i>Ameiurus</i>	8	6	39	4	57	20	50	14	36	20	5

be due to an acclimatization to low oxygen. It is fully as probable, however, that the change was due to the fact that in the low oxygen aquarium the fishes formed the habit of swimming much of the time at the surface and thus in the experiment the gradient would not be noticed. This habit of swimming at the surface was necessary because the fishes died if confined below the surface of low oxygen water.

We had only a low oxygen stock of *Umbras* for boiled water experiments but their reactions are similar to those given by other fishes from high oxygen. Low oxygen *Ameiurus* showed about the same preference for the tap water in one watched experiment as did the high oxygen stock. A limited number of observations in the glass boxes showed the high oxygen stock

to be more active and probably more stimulated by the experimental conditions. In the long experiments *Ameiurus* showed a preference for the boiled water end but the high oxygen stock showed the same in two out of three trials.

b. Boiled water with oxygen added

These experiments were essentially like the preceding ones but oxygen was added to the boiled water end so that the amount in solution was equal to that in the tap water. Thus the fish encountered changes in water as described for the boiled water gradient experiment with the exception of oxygen. The controls were different from the controls of other experiments, in that boiled water was introduced into one end of the control tanks and tap water into the other. The control was then an experiment and the amount of reactions to factors other than oxygen was determined by comparing the experiment and control. The general results are shown in table 15. *Lepomis* (experiment 80) *Hybopsis* (experiment 78) *Catostomus* (experiment 82) and *Notropis* (experiment 77x) showed a time preference for the boiled water with oxygen added, but the average time preference was for the tap water, these being exceptions. The turnings back in the gradient were not accompanied by any of the characteristic reactions described for carbon dioxide. As a rule the fishes turned back from the boiled water with oxygen added, oftener than from the tap water, but *Notropis*, experiment 77 A, furnishes an exception.

In the controls (really experiments with boiled against tap water) the fish gave practically the same reactions that have already been discussed in treating of the reactions to the boiled water gradient (p. 241). In general, the time preference was much greater for the tap water while the number of turnings was about the same for each end. The sharper reaction as shown in the matter of time, shows the effect of oxygen upon the reactions of fishes.

TABLE 15

Showing the reactions of fishes to boiled water with oxygen added to balance the oxygen of the tap. The gradient consisted of a few parts per million of calcium, et cetera, 15 cc. of nitrogen, 2 cc. of free and 1 cc. of half-bound carbon dioxide. Data arranged as in the preceding tables. Controls were gradients between tap and boiled water with no oxygen added, so that they are like the experiments tabulated in table 11, p. 243.

EXPT. NO.	SPECIES	NO. SIZE	O ₂ IN CC. PER LITER		CROSS- ED CEN- TER	TIME	PER CENT OF TIME IN HALVES T, TAP; B, BOILED WATER				TURNED BACK FROM WATER AS INDICATED				TEMP. DEG. C.				
			Expt.				Control						Expt. Control						
			Tap	Boiled + O			Tap	Boiled	Expt.	Control	T	B + O ₂	T	B		T	B + O ₂	T	B
			T	B + O ₂			T	B	T	B + O ₂	T	B	T	B		T	B	T	B
83 X	Micropterus	1 9	9.16	9.91	9.16	4.16	13	41	20	85	15	65	35	4	11	3	10	8-8.5	
80	Lepomis	3 5	9.16	9.91	9.16	4.16	48	13	20	46	54	54	36	19	19	3	0		
77 A	Notropis	3	9.16	9.91	9.16	2.83	86	52	20	54	46	64	36	9	4	14	30	9	
77	Notropis	3	9.16	9.91	9.16	2.83	31	73	20	46	54	48	52	1	10	22	30	9	
79	Notropis	3	9.16	9.91	9.16	2.83	106	56	20	55	45	56	44	10	13	12	18	9	
78	Hybopsis	3	9.16	9.91	9.16	2.83	19	76	26	31	69	84	16	2	2	7	9		
83	Ambloplites	3 4-8	9.16	9.91	9.16	2.83	40	109	20	60	40	63	37	1	9	1	9	8-8.5	
83 A	Ambloplites	3 4-8	9.16	9.91	9.16	2.83	88	52	20	65	35	73	27	4	10	1	13	8-8.5	
81	Abramis	2	9.16	9.91	9.16	2.83	23	62	20	59	41	84	16	1	4	0	6	9	
82	Catostomus	3	9.16	9.91	9.16	2.83	14	48	20	48	52	55	45	6	3	3	17	8	
71	Hybopsis	3	9	9	9	2.4	310	44	(E45) (C30)	67	33	57	43	5	30	4	11	8	
Average.....						72	35		56	44	63	37	5.5	10	6	13			

¹ Fish stayed in boiled water plus oxygen. After finding the tap water they spent most of the time in that end.

² Fish did not encounter tap water until the end of the first five minutes. After the tap water was encountered the fish spent 62 per cent of the time in that end.

³ Fishes driven asymmetrically.

⁴ No evidence of a reaction.

c. Boiled water and carbon dioxide

The results of these experiments are shown in table 16. The oxygen content of the boiled water was less than 1 cc. per liter as it flowed into the tank. The carbon dioxide content was kept as nearly as possible at 50 cc. per liter at the boiled water and 3 cc. per liter at the tap water end. As shown by the table and by the graphs, chart 4, the reactions of all the fishes were very decided. In every case they showed a strong time preference for the tap water and turned back from the treated water much oftener than from the tap. All the testing (backing-starting)

TABLE 13

Showing the reactions of fishes to a gradient of boiled water accompanied by high carbon dioxide. Data arranged as in preceding tables. Controls in tap water. Gradient between tap and boiled plus carbon dioxide.

EXPT. NO.	SPECIES	NO.	CO ₂ IN CC. PER LITER		CROSS-ED CENTER	TOTAL TIME IN MIN.	PER CENT OF TIME IN HALVES				TURNED BACK FROM						
			Low	High			Expt.	Control	Expt.		Control		Expt.	Control	Expt.		
									L.O ₂ High CO ₂	H.O ₂ Low CO ₂	Corresponding				High CO ₂	Low CO ₂	Corresponding
											High CO ₂	Low CO ₂					
57	Lepomis	3	3	50	28	64	20	6	94	66	34	24	2	5	2		
57 A	Lepomis	3	3	50	29	5	20	17	83	66	34	28	9	0	0		
60	Notropis	3	3	50	6	57	20	1	99	50	50	80	0	6	5		
61	Ameiurus	2	3	50	6	33	20	5	95	33	67	10	0	1	4		
62	Umbra	2	3	50	13	15	20	12	88	67	33	9	1	1	6		
59	Catostomus	3	3	50	8	3	20	4	96	48	52	25	0	0	0		
55	Ambloplites	3	3	50	20	5	20	11	89	37	63	16	0	0	0		
55 A	Ambloplites	3	3	50	5	17	20	5	95	23	77	9	0	1	1		
58	Abramis	3	3	50	37	38	20	8	92	24	76	11	0	0	0		
58 A	Abramis	3	3	50	19	37	20	8	92	41	59	5	0	1	4		
56	Hybopsis	3	3	50	33	30	20	3	97	63	37	7	0	5	0		
56 A	Hybopsis	3	3	50	20	32	20	8	92	68	32	3	0	0	0		
Average					19	26		7	93	49	51	19	1	2	2		

coughing, gasping and gulping reactions were given with greater frequency and greater intensity than in any of the other experiments.

When observed in the glass boxes, Hybopsis, Lepomis Ameiurus, Umbra and Ambloplites showed great disturbance in the boiled water with carbon dioxide added. There was uniformly greatly increased activity, increased opercular movements, and special reactions, such as gulping, rising to the surface, et cetera, as described for boiled water alone. These were accompanied by some lack of coördination and in one case (Ambloplites) by falling on the side.

We have in the experiments good evidence that fishes turn back from waters high in carbon dioxide and low in oxygen with precision and vigor. Also that if they enter such localities, they cannot behave normally and may soon die. When we compare these results with those on boiled water or oxygen and with the results on carbon dioxide alone we see that carbon dioxide is the most potent factor yet studied in this series of experiments.

d. Acid and ammonia

The only two species of fishes used reacted to a combination of acetic acid and boiled water (table 17, chart 3) in a manner comparable to the response given to boiled water and increased carbon dioxide. Since the reactions were so clearly parallel, no further experiments were tried. This acidity was far greater than when carbon dioxide was used (table 16) and since the reactions were not markedly different, it is probable that the fish react to some factor other than acidity in the case of the carbon dioxide. Thus it would seem that the narcotic action of carbon dioxide is more important in fish reactions than its action as an acid.

TABLE 17

Showing the reactions of fishes to boiled water accompanied by acetic acid. Data arranged as in preceding table. Controls in tap water. Experiments with gradient between tap and treated water.

EXPT. NO.	SPECIES	NO.	ACID IN GRAMS PER LITER		CROSSED CENTER		TIME IN MIN.	PER CENT OF TIME IN HALVES			TURNED BACK FROM BOILED (B) ACID (TAP)				
			L	H	Expt.	Control		Expt.	Control		Expt.	Control			
									B + acid	Tap		Cor. acid	Tap	B + acid	Tap
41	Abramis.....	2	3.36	12	28	22	30	15	85	56	44	29	0	1	0
42	Ameturus.....	2	4.35	12	67	142	40	19	81	56	44	26	5	21	13
Average.....					48	82		17	83	56	44	28	3	11	7

The reactions to ammonia and boiled water are shown in table 18. Although their reactions to boiled water were marked Abramis did not react to boiled water and ammonia in a concentration which caused them to turn on their sides after an hour or more. With the higher concentration this was true also but more markedly since the fishes showed a time preference for the high ammonia, though free to go back and forth. The three Abramis, died after from nineteen to twenty-three minutes. The only definite response given was the testing reaction which occurred very often in both ends of the tank. A single Notropis put in with the Abramis died after thirty minutes.

TABLE 18

Showing the reactions of fishes to a gradient of boiled water with ammonia added. Data arranged as in preceding tables. Controls in tap water. Gradient between tap and treated water.

EXPT. NO.	SPECIES	NO.	AMMONIA IN CC. PER LITER		CROSSED CENTER		TIME IN MIN.		PER CENT OF TIME IN HALVES				TURNED BACK FROM (NH ₄ OH + BOILED) OR TAP			
			Low	High	Expt.	Control			Expt.		Control		Expt.		Control	
									Correspond							
									NH ₃	Tap	NH ₃	Tap	NH ₃	Tap	NH ₃	Tap
36	Abramis.....	3	1	85	29	27	30	42	58	58	42	0	0	1	0	
39	Ameiurus.....	2	1	102	44	108	30	60	40	62	38	2	3	18	10	
Average.....					37	68		51	49	60	40	1	2	10	5	

Ameiurus showed some signs of stimulation, such as gulping but gave no movements which tended to bring the fish into better conditions. As in Abramis, no avoiding or regulatory reactions were given. After going back and forth for twenty-nine minutes, the two fishes came to rest, one in the high ammonia and low oxygen, the other in the low ammonia and high oxygen. The former was apparently dead at the end of forty minutes though it recovered after a week or more in clear water. The one in the lower ammonia lived and showed no sign of having been affected.

While these two species probably rarely encounter acid media except carbonic acid, they react to the acid in much the same manner as to carbon dioxide. This result is in accord with much experimental work in animal behavior.

Fishes must encounter ammonia in very weak concentrations quite often in primeval nature, but the species studied in these preliminary experiments, appear to be unable to react to the concentration used, at least when it is accompanied by low oxygen content. Low oxygen accompanies ammonia in sewage and if the results obtained with these fishes are the rule, the relation of fishes to ammonia and low oxygen is a life or death matter.

4. COMPARISON OF REACTIONS

We have discussed the reaction of several species of fish to various factors and the combination of these factors, without especially considering the differences of reactions given by fishes of different species or unlike age, to any one set of factors. In this section we will discuss comparative aspects.

a. Degree of reaction to the different factors

Table 19 shows the average reactions of the controls. It indicates that some of the fishes, as *Ambloplites* and *Catostomus*, sometimes spent a greater part of their time in one end of the control tank when the two ends were alike as far as we could know. The majority of the controls show almost a balanced time average, and in four of the ten species studied this is exactly balanced. The number of turnings is more variable and in some

TABLE 19

*Showing the average control responses for each species. The turnings are given in percentage of the total number and a rating is given of the degree of asymmetry of response or the apparent preference for one end. The ratings are obtained by subtracting the percentages given for the two ends and dividing their sum by two. For example, in the case of *Ameiurus*, 49 from 51 gives 2 and 42 from 58 gives 16. Since the turnings do not agree with the time preferences they must be considered of the opposite sign. Adding ± 16 and ± 2 gives ± 14 which, divided by 2 gives the rating as ± 7 . This is a numerical expression of the time spent and the turnings from each end when they are considered of equal value.*

SPECIES	NO. OF CON- TROLS	AVE. NO. OF FISH	AVE. CROSS- ING CENTER	AVE. TIME	AVE. PER CENT OF TIME		AVE. PER CENT OF TURNINGS		RATING
					West end	East end	From west end	From east end	
<i>Ameiurus</i>	7	2	60	31	51	49	58	42	± 7
<i>Abramis</i>	15	3	77	26	51	49	62	38	± 11
<i>Hybopsis</i>	11	3	47	22	50	50	50	50	± 0
<i>Ambloplites</i>	8	3	30	31	42	58	22	78	± 20
<i>Catostomus</i>	6	3	43	25	37	63	43	57	± 6
<i>Umbra</i>	5	3	42	32	46	54	50	50	± 4
<i>Notropis</i>	10	3	45	22	47	53	45	55	± 8
<i>Lepomis</i>	7	3	43	29	42	58	50	50	± 6
<i>Etheostoma</i>	2	3	58	40	51	49	64	36	± 13
<i>Micropterus</i>	2	1.5	17.5	27.5	53	47	0	0	± 18

cases counterbalances the apparent time preference for one end. To be consistent, there must be more turnings from the end in which the least time is spent. When this does not occur, the apparent time preference is neutralized to a greater or less extent, as the case may be. The figures in the column marked 'rating' in table 19 are attempts to express numerically the intensity of reaction when both turnings and time preferences are considered. The ratings were obtained by adding the difference in the percentage of time spent in the two ends, to the difference in the percentage of total number of turnings. This sum divided by two gives the rating average. In the experiments where the fish avoided the tap water or the water nearest like that in which they had been kept, they were rated as negative. Thus the rating for *Ameiurus* from the low oxygen stock in relation to the boiled water is in favor of the boiled water end of the experiment, so they are rated as +18.

In the experiments the degree of negative reaction to the various factors and combinations of factors is shown in table 20. The ratings of the boiled water, oxygen, et cetera, group are very conservative, as the averages upon which they are based include cases where fishes reacted by coming to the surface, et cetera, and which neutralized the reaction in experiments where our methods are effective (p. 246). In some cases few experiments were run with a given set of conditions because the reactions were very decided, so that further experiments were unnecessary for the purpose of this paper. Considering the averages as they stand, it will be noted that the greatest vigor of reaction is shown to carbon dioxide in boiled water and to the combination of carbon dioxide and boiled water with tap water at the other end of the gradient. The reactions to carbon dioxide in tap water and that to acetic acid in boiled water are about equal and stand next in rank. Of the experiments where carbon dioxide and acetic acid are not concerned, the reaction is most definite to an oxygen gradient in boiled water. Fishes vary greatly in the vigor of their reaction to both boiled water and to oxygen alone.

Hybopsis proved most sensitive of all the fishes tried. The graphs in chart 4 show the manner of reaction and the movements

TABLE 20

Showing relative vigor of reaction by species and factor rating obtained as in table 19. Except where otherwise specified the ratings are negative; in other words the different figures signify relative avoidance of the kind of water mentioned first except in the cases of the reactions of the low oxygen stock to boiled water and the reactions to ammonia where signs indicate the reaction to the treated water. Number of experiments given applies to the column following.

SPECIES	NO. CONTROLS	ACCIDENTAL PREFERENCE FOR ONE END OF CONTROL	NO. EXPTS.	CO ₂ + BOILED WATER VS. BOILED	NO. EXPTS.	CO ₂ + BOILED WATER VS. TAP	NO. EXPTS.	ACETIC + BOILED WATER VS. TAP	NO. EXPTS.	NO. EXPTS.	CO ₂ IN TAP WATER VS. TAP	NO. EXPTS.	N ₂ + (O ₂ IN BOILED WATER VS. BOILED WATER + O ₂)	NO. EXPTS.	BOILED WATER VS. BOILED WATER + O ₂	NO. EXPTS.	BOILED WITH O ₂ BALANCED VS. TAP WATER	NO. EXPTS.	LOW O ₂ STOCK TO BOILED WATER VS. TAP	NO. EXPTS.	AMMONIA + BOILED VS. TAP	RATING ON A BASIS OF 5 CC. OF CO ₂	AVERAGE RATING FOR CO ₂ + BOILED, CO ₂ IN TAP, (O ₂ AND BOILED)
Hydrae...	11	±0	2	97	2	94	2	94	2	3	87	2	25	3	67	33	33	1	NO. EXPTS.	1	±8	33	17
Notopis...	10	±8	1	99	1	99	1	99	1	3	71	1	40	3	39	8	8	1	NO. EXPTS.	1	±8	74	63
Microceres...	2	±3	2	92	2	92	2	92	2	2	46	1	39	2	37	1	1	1	NO. EXPTS.	1	±8	30	82
Amphipodes...	8	±20	2	92	2	92	2	92	2	2	46	1	39	2	37	1	1	1	NO. EXPTS.	1	±8	33	52
Lepomis...	7	±6	2	92	2	92	2	92	2	2	74	2	18	2	18	14	4	1	NO. EXPTS.	1	±8	44	44
Cerastomus...	6	±6	2	92	2	92	2	92	2	2	74	2	18	2	18	14	4	1	NO. EXPTS.	1	±8	53	53
Eucostoma...	2	±13	2	92	2	92	2	92	2	2	74	2	18	2	18	14	4	1	NO. EXPTS.	1	±8	33	33
Abramist...	13	±11	2	83	2	91	1	85	1	3	60	3	73	1	73	1	39	1	NO. EXPTS.	1	±8	23	75
Anchirus...	7	±7	1	95	1	95	1	95	1	1	69	1	38	1	38	1	39	1	NO. EXPTS.	1	±8	53	53
Umbra...	5	±4	1	78	1	78	1	78	1	2	69	2	38	2	38	1	39	1	NO. EXPTS.	1	±8	55	55
Total...	78		182			701		143			557		114		311		182						
Average...	±8		91			88		71			70		38		35		26						

¹ Not in accord with table 11; see p. 243, especially note 3, and bottom of p. 255.

of this fish to the various factors. As shown by the chart, *Hybopsis* clearly avoids water which has lost some of its salts, carbon dioxide and nitrogen (p. 240), and reacts a great deal more vigorously to water which has also lost most of its oxygen. However the reaction to an oxygen gradient in boiled water is less definite than to tap against boiled water. The reactions to carbon dioxide are all definite, being most so to carbon dioxide in boiled water.

Table 20 not only gives relative vigor of reaction when read from right to left but enables one to compare species by reading from top to bottom, in so far as the various factors have been worked. *Notropis* and *Abramis* stand second after *Hybopsis*. The data are far too incomplete and the experiments too few in number to justify general comparison. The column at the extreme right suggests the reactions of the different species to combinations of oxygen and carbon dioxide. The amounts of carbon dioxide used were much higher than the animals commonly encounter in nature, so that these figures could not be used for ecological ratings and comparisons even if there were enough experiments to justify the attempt. The ratings on the basis of a gradient of 5 cc. of carbon dioxide per liter given in the column to the left of the last would come nearer to rating the fishes according to their distribution in clear, well-aërated water and stagnant and foul waters. The reactions to oxygen are clearly greater than the rating of accidental preference for one end or other of the control, while the reactions to the effect of boiling, with oxygen added to balance, are variable and the rating is less than the errors of the controls for *Lepomis*, *Notropis* and *Catostomus*.

When compared with the habitat preference data of Forbes and Richardson ('08, pp. 79-85), we are unable to see definite correlations between the occurrence of fishes with reference to size of stream or pond; or with reference to current or kind of bottom. However, it appears significant that *Hybopsis*, which proved to be the most sensitive fish in these experiments, is shown to have the most limited habitat preference of any of the fishes studied. Since the environment of fishes is a complex of many factors we cannot expect correlation to be possible until experimental study has been carried much farther.

b. Adult and juvenile fishes

We have had only a little opportunity to note the differences in behavior of adult and juvenile fishes. Wiegelt ('85) found that young fishes were more sensitive to ammonia than adults. As a rule, in our experiments the younger and smaller fishes were more easily affected by the various stimuli employed than the adults of the same species. In a general way, the adults and where used, the young also of the hardier fishes (*Abramis*, *Umbra*, and *Ameiurus*) react clearly in a negative manner to carbon dioxide, acid, and with less vigor, to lack of oxygen. In all probability the adults of the food and game fishes react in a manner comparable to the young, but with the equipment at hand, we were unable to obtain recordable results.

Among other observations of this kind, a large *Lepomis* was placed in the tank with the smaller individuals in an experiment with a carbon dioxide gradient in tap water. During the entire period of experimentation, the young fishes went back and forth, both turning back from the center and spending a longer time in the tap water. The large fish came to rest in the carbon dioxide end, and although clearly affected, as shown by gulping and rising to the surface, remained in the carbon dioxide for twenty-six-and-one-half minutes before encountering the tap water end. During the next seven minutes, it remained most of the time in the tap water but after that went back and forth for seven minutes at a very rapid rate and without stopping in either end. In a thirty-minute control, the same fish did not cross the center at all. The greater speed of this large fish probably carried it to the end of the tank before it could be expected to turn, after having been affected by entering the carbon dioxide water. In such a case it could not have been possible to obtain recordable results. The smaller size of the tanks, in proportion to the size of the fish probably makes the surroundings much more unnatural. In connection with the study of adult fishes, we conclude that for the adults of the food and game fishes, the tanks should be about three to five times as long as ours and probably twice as wide and deep. We must, however, leave the matter as a special subject for investigation.

VI. GENERAL DISCUSSION

Previous to this series of almost a hundred experiments, we spent much time in devising means of applying the gradient method of experimentation to our particular problems. By the methods used we have ascertained that certain fishes react clearly to varying amounts of oxygen, carbon dioxide, acids, and to the general effect of boiling. The possibility of more accurate, more detailed, and more comprehensive work is evident, and is now being undertaken in this laboratory. These experiments were planned only as an introduction to the subject.

We have noted that each species has differences in details of movement and of resting but we have made no attempt to present here a detailed statement of these differences. While recognizing these differences, we have found a clear correspondence in the general avoiding reactions given under certain conditions. Such reactions are gasping, rising to the surface, increased respiratory activity, and turning back from the disturbing condition of the water. These general characters of reaction clearly dominate over the more specific details in the matter of successful avoidance of otherwise stimulating conditions. This makes specific peculiarities of minor significance in the success of fishes and points clearly to physiological characters comparable to generic, family, and ordinal characters but which bring together fish in no wise taxonomically related. These groupings on the basis of physiological or ecological characters have no relation to groupings made on the usual bases of taxonomy. The majority of investigators are commonly impressed with the detailed structural characters of the organism and such peculiarities of behavior as go with them, quite forgetting the physiological processes and groupings which are clearly general in the sense that they belong to whole groups of organisms (Shelford '12).

While the possibility of groupings is clearly suggested by differing vigor of reaction on the part of different species, it is not possible to outline such groups definitely on the basis of our data, for reasons outlined on page 257. Furthermore such groupings may have to be based primarily on the reactions during the breeding season. Reactions to solutes must be considered in

connection with the breeding period and breeding condition, if we are to explain the distribution of fishes in nature and make our groupings ecological. The number of ecological factors is clearly great. The entire life history of the species must be studied both by experiment and by observation and *first*, as we believe, with particular reference to the *physiological characters of the greater magnitude*, leaving the specific aspects until later. For these reasons we have studied several species, hoping to get a hint concerning these physiological characters of the higher order. That is, we were hunting for the generalities of behavior that would apply to ten species of fishes widely distributed taxonomically, rather than the specific details of the behavior of any one species. Had we chosen to work entirely on Abramis, the abundant and easy species, we could have completed a detailed bit of work of a type highly approved by investigators but which would clearly have led us into error, because of the specific peculiarities of the species. These peculiarities would have led to incorrect interpretations of the behavior of the individual fish and to a much greater error if the data had been used as a basis for generalizations. Thus we would have accumulated a mass of details of doubtful application to current problems, however interesting they might have been of themselves. Had we studied only darters we should have erred in a still more dangerous direction.

Turning to the practical application of our conclusions to current biological problems, we find that they fall under three main heads. First, the economic and distribution problems; second, the problems of fish physiology; and third, the problems of behavior and psychology. From the economic point of view, it appears to us from these experiments, that emphasis has been wrongly placed upon environmental factors as matters of life and death to the fishes concerned. Clearly, fishes are often absent from accessible situations which upon inspection appear favorable, and where an examination of the water shows conditions entirely compatible with life. It appears also that the importance of oxygen in determining the distribution of fish, has been too much emphasized. The oxygen optimum of all the

fish studied, as was that of the fish studied by Duncan and Hoppe-Seyler ('93), is clearly low. Fishes react to oxygen gradients, though usually indefinitely.

On the other hand, the importance of carbon dioxide in fish distribution has been largely overlooked. It is significant that even in tap water, all the fish tried reacted very definitely to an amount of carbon dioxide that is scarcely greater than that often found in ponds. Increased carbon dioxide is usually accompanied in nature by low oxygen and it is to the combination of lack of oxygen (boiling) and increased carbon dioxide, that the fish react most definitely. We accordingly feel justified in stating that the carbon dioxide content of the water (not excessively alkaline) is the best single index (Shelford and Allee '12) of the suitability of water for fishes. Half bound carbon dioxide may be of some importance in alkaline waters but our evidence tends to show that in neutral or acid water it has little effect. Certainly in survey work designed to determine the suitability of water for fishes, the determination of the carbon dioxide content and the study of the conditions necessary for breeding (Shelford '11) should not be omitted.

From the standpoint of the physiology of fishes we have contributed little but have added some confirming data to the observation that adult fishes are less sensitive than juvenile ones of the same species, and that carbon dioxide acts as a narcotic and in small quantities stimulates the respiratory center (Reuss '10). The experiments indicate also that the fishes detect differences in the character of water but the localization of the reception of such stimuli has not been studied.

From the standpoint of the behavior and psychology of fishes, we note that fishes are able to react to stimuli by simple turnings back, and that as a rule, they remain longer in water which does not clearly influence the details of their activities. That the formation of associations may enter into the latter type of reaction and perhaps also into the former is suggested by the more decided avoidance of treated water which comes with repeated entrances into it. It seems, however, that such results may possibly be otherwise interpreted. It is possible that time is

required for the fishes to become affected by the lack of oxygen, carbon dioxide, et cetera, and that when the system has once been affected, for example, by carbon dioxide, a slight increase may have a more pronounced effect than at first when the blood supply is relatively free from this substance.

VII. SUMMARY

1. In the experimental control of gases, several and not one factor are commonly varied; the varying of single factors is unusually difficult, being essentially impossible, when gas is bubbled through water (p. 210).

2. Fishes are clearly affected by lack of oxygen; species usually die in the order of their relation to these factors in nature (p. 223).

3. Carbon dioxide, in concentrations probably used to produce reversals of reaction in some of the invertebrates, is poisonous to fishes, producing death very quickly (p. 224).

4. Fishes are not seriously affected by high nitrogen except when gas is in excess under one atmosphere pressure and comes off in bubbles on rough and warm objects; under these conditions gas bubble disease occurs (p. 222).

5. Fishes react negatively to a gradient of decreasing salts, nitrogen, and 1.5 cc. per liter of carbon dioxide, in combination; to a decrease in oxygen and other effects of boiling in combination; to carbon dioxide, to carbon dioxide in combination with the effects of boiling, and to carbon dioxide in boiled water at both ends of the experimental tank. The precision and definiteness of the reaction is indicated by the order in which the factor and combinations are given the most definite reaction being to carbon dioxide (p. 229).

6. The negative reaction of the fishes is evident through longer stays in the tap water end of the gradient tanks, by turnings back from the center, by risings to the surface, or by any combination of the three (p. 231).

7. Such reactions are accompanied by backing-starting reactions, coughing, gasping and gulping, directly proportional to the degree of avoidance of the treated water (p. 231).

8. The two species tried reacted negatively to a gradient of acetic acid in boiled water but some of the species did not react to ammonia in concentrations which produce death (p. 252).

9. Some fish establish 'preferences' or apparent preferences for one end of the experimental tanks for reasons obviously due to the experimental conditions (pp. 246, 261).

10. There is a large similarity of reaction among fishes that differ widely taxonomically, which indicates the possibility of groupings which are of generic, ordinal, or family value, but which bear no relation to existing taxonomy (p. 259).

11. The carbon dioxide content of the water is probably the best single index of the suitability of the water for supporting fishes (p. 261).

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EXPERIMENTS CONCERNING THE SEXUAL DIFFERENCE IN THE WING LENGTH OF *DROSOPHILA AMPELOPHILA*

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TWO FIGURES

Two of the forms of the fruit fly, *Drosophila ampelophila*, which have been isolated recently by Prof. T. H. Morgan are distinguished from the normal by lesser wing length. One, called 'wingless' in Professor Morgan's papers, really possesses vestiges of wings which appear to consist largely of modified basal portions of normal wings. The other, called 'miniature,' possesses all the veins of the normal wing in approximately normal condition but the wing is only about two-thirds the normal length.

'Winglessness' is recessive to normality according to the simple Mendelian formulae. In F_1 all individuals, both male and female, are hybrid no matter which parent bears the abnormal character. The 'miniature' wings are also recessive but are sex limited in their inheritance. If the mother have miniature wings and the father be normal, only the females of F_1 will be hybrids while all the males will be pure recessives. In the reciprocal cross the females will again be hybrids but the males will be pure dominants. The reasonable explanation which Professor Morgan has advanced of these phenomena is that the factor for miniature wings (using such an expression in lieu of a better) is contained in, or in some way connected with, that chromosome of which the female possesses two and the male but one, while the factor for winglessness is connected with something which is shared equally by both sexes. This idea is shown diagrammatically in figure 1 in which the composition of pure stock of the three forms and that of two of the cross are shown, the *X*-chromosomes being represented by squares.

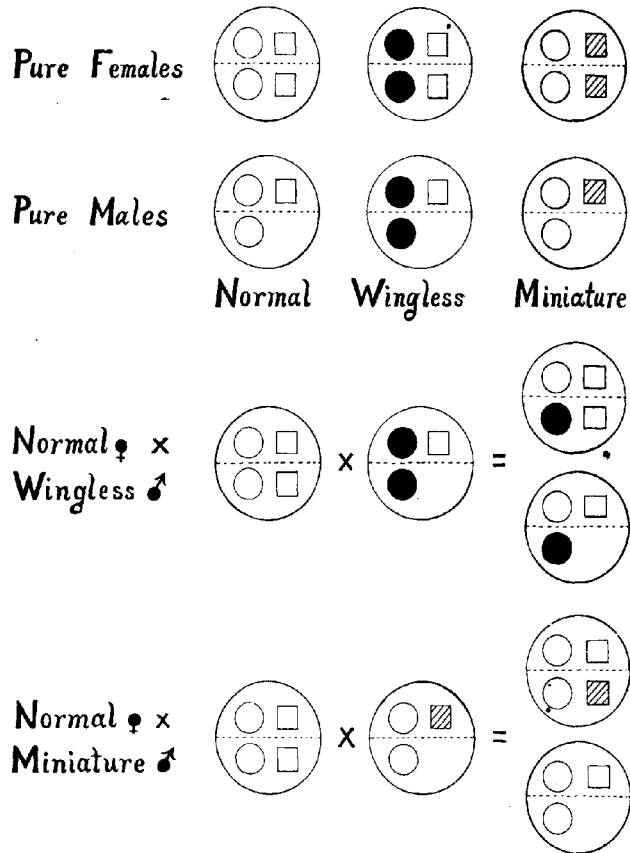


Fig. 1 Theoretical composition of zygotes. The squares represent the X-chromosome. The small circles represent the rest of chromosomes. Where they are plain their composition is supposed to be normal.

TABLE 1
Average dimensions. See text.

		NORMAL ♀ X NORMAL ♂	NORMAL ♀ X WINGLESS ♂	NORMAL ♀ X MINIATURE ♂
Wing.....	♀	71.404±0.180	65.644±0.210	70.932±0.139
	♂	64.025±0.137	58.568±0.151	64.737±0.149
Femur.....	♀	73.059±0.162	69.695±0.236	71.045±0.157
	♂	69.194±0.170	68.053±0.160	69.651±0.174
Wing : Femur.	♀	99.199±0.128	93.851±0.156	98.409±0.145
	♂	91.783±0.116	85.900±0.175	92.767±0.133
♀-♂		7.416±0.173	7.861±0.234	5.642±0.197

It will be seen from table 1 that the mean wing length of homozygous normal females is considerably greater than that of similar males—their brothers. The average length of the middle femur is also greater in the females than in the males but the sexual dimorphism with respect to wing length is relative as well as absolute, as is shown by the fact that the ratio of wing to femur among the females greatly exceeds that among the males, the difference between the averages being nearly fifty times the error of the difference. Even so, this would not prove the existence of a fundamental sexual dimorphism if there were a tendency for generally large flies to have the ratio large. Without entering the maze of spurious correlation caused by using indices, we can see from the regression lines (fig. 2) that in both sexes, but especially in the male, there is a tendency for the wing to get proportionately smaller as the general size of the insect, as measured by the size of the middle femora, increases. The dimorphism is therefore real. The sexes are built on different plans.

It is in all ways probable that there is a large complex of factors concerned in the development of a normal wing. It is possible that the abnormal forms considered here are caused by the dropping out of certain of the factors from this complex. It would seem that the normal females get a double dose and the males but a single dose of those factors of the normal complex which are connected with the X-chromosomes. It is well known that many factors do not cause as great a somatic development when in a simplex condition (for example, heterozygous), as these

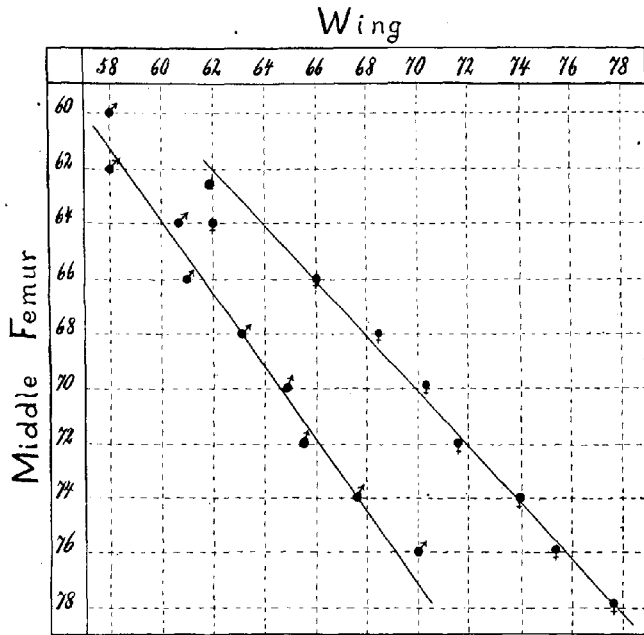


Fig. 2 Regression lines for homozygous normal flies.

same factors cause when in a duplex or homozygous condition. May it not be, then, that the greater wing development in normal females is caused by their getting a double dose of certain germinal elements, one dose for each X-chromosome, while the males get but a single dose? The following experiments were tried in the hope of getting some answer to this question.

All the flies in the three sets of experiments were reared at the same time, given an abundance of food from the same jar of fermenting banana, kept in the same sort of bottles which were placed side by side on the table. Furthermore, the flies for each experiment were the offspring of several score of freely interbreeding parents and were reared in a number of different bottles so that the chance variations of ancestral bias among the parents and

of environmental effects from bottle to bottle would tend to be equalized in the three sets of offspring. This care was taken because of the marked influence environmental conditions, at least, have upon general body size.

Delcourt and Guyenot¹ have suggested that in my note on the failure of disuse to decrease wing length, I should have given the ratio of wing length to body length. It did not, and still does not, seem to me to be necessary since all the factors involved, except disuse of wings, tended to decrease the general body size as much as or more than that of the wing. In this paper, however, it does seem desirable to have some other character with which to compare the wing. Among all that are feasible the body length is the worst because it may change from hour to hour in the living insect, can be measured only with great difficulty and changes greatly after death. I have used the length of the middle femora because it has none of these disadvantages. The wings and legs used here were removed from freshly etherized flies and immediately mounted in balsam. Measurements were made and are recorded in units of $\frac{1}{32}$ mm. for the wings and $\frac{1}{16}$ mm. for the femora.

Taking up first the offspring of normal females x wingless males, we find that, while the wing is approximately of normal length in both sexes, both it and the femora are significantly smaller than in the homozygous normal material. This may possibly be an environmental effect which was not entirely avoided by the cultural methods used. However, the ratio of the wing to femur is also significantly smaller in both sexes. It is smaller even though, as was pointed out above, there is a tendency for small flies to have the ratio larger than in the case of large flies.

¹ Bull. Scient. France et Belgique, 7th Serie, tom. 45, no. 4. In a general denunciation of all the work hitherto done with this insect, they deplore the fact that the results have been obtained without the extreme refinements of bacteriological and physiological methods which they recommend. Their criticisms, insofar as they have any value, can be applied only to the study of fluctuating variants such as the characters considered here. All attempts to get heritable abnormal venation or such forms as wingless and miniature by purposely using extreme environmental conditions have failed. It is, therefore, absurd to lay stress in such cases upon the slight variations of environment from bottle to bottle.

The heterozygous wings are to all appearances normal but they are really not relatively as long as normal.

As is indicated in figure 1, both sexes of these flies have only one dose of that part of the normal wing complex which presumably dropped out to give a wingless fly, whereas both sexes of normal flies have two doses. In wingless flies, and hence in half of each zygote from which these hybrids came, something has been changed, at least, so that the tendency to develop the wings to normal proportions has been lost. It is probable that the result of this experiment, a phenomenon usually referred to as 'incomplete dominance,' is due to this cause.

However that may be, the interesting point for the present discussion is that the sexual dimorphism has not been changed. Environmental effects are practically ruled out here since the males and females of a given experiment grew up together. Normal females have a wing-to-femur ratio 7.42 greater than the males and in these flies it is 7.86. The difference of 0.44 is less than twice the probable error and certainly is not significant. On the hypothesis stated above, this is what is to be expected since the germinal changes are alike in the two sexes.

Conditions are theoretically quite different in the cross between normal females and miniature-winged males. It will be seen from figure 1 that normal females get two doses of that part of the wing complex which is connected with the sex chromosome whereas the males get but one. In order that miniature wings may appear, this part of the complex must be changed, either by the dropping out of a factor or in some other way. In the cross just mentioned the male offspring are, according to theory, perfectly normal in their germinal make up. Their single X-chromosome has its full share, and no more, of wing factors. The female, however, has only one normal X-chromosome. The other either lacks a factor or, less probably, has a new modifying factor. At any rate the second X-chromosome is not equipped for full wing development. Hence the sexes are more nearly alike in their germinal make up than are the normal. The results show that they are also more nearly alike in their somatic condition.

The difference between these males and the normal males with respect to the wing-to-femur ratio must be considered as an environmental effect since they are supposed to be germinally the same. It is 1.01 ± 0.15 and is doubtless significant in spite of the precautions taken. However, as was pointed out above, we escape even this difficulty when comparing brothers and sisters since there is no evidence and it is not believable that a given environmental condition will operate to increase the relative size of the brothers' wings and decrease that of the sisters' as is the case in this cross. The sexual difference is only 5.64, that is, 1.78 ± 0.25 less than normal. The sexual difference in these flies is still nearly thirty times its probable error but in the normal lot it is about fifty.

An explanation of this remaining and still considerable sexual difference is not difficult of framing on the hypothesis here followed. In fact, a wiping out of all sexual difference would have proved too much. It is only recently that any sex-limited characters have been known. It is more than likely that there are many factors concerned in wing development. Certainly all the factors have not been isolated since wings have not been entirely done away with even in the so-called wingless strain. We are free, then, to postulate that it is these remaining factors which cause the remaining sexual dimorphism of wing length.

Therefore it seems that, while proof is lacking, indications have been found that the greater wing development in the normal females of this fly than in the males is due to the females getting two sets of those wing factors which are connected with the X-chromosome while the males get but one, the double set causing a greater somatic effect than the single.

THE SEX OF A PARTHENOGENETIC TADPOLE AND FROG

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THREE FIGURES

Bataillon has shown that the unfertilized egg of the frog can be caused to develop by puncturing it. Last spring we tried the experiment in a large number of eggs of various species of anura.

The females were separated from the males, carefully washed with water and with alcohol, and then opened. The eggs were taken out of the uterus with sterilized instruments without coming in contact with the surface of the frog. About 20 per cent of the unfertilized eggs were kept as controls and 80 per cent were punctured. A few eggs were fertilized with sperm. Not a single unfertilized control egg segmented or developed. The number of unfertilized eggs which began to segment after puncture was greater in the wood frog than in the leopard frog, and amounted in the most favorable cases to about 40 per cent in the former. Only 2 of about 10,000 punctured eggs of the wood frog reached the tadpole stage, but these died before they were able to swim. The percentage of eggs of the leopard frog which reached the tadpole stage was greater. From 700 punctured eggs of the southern leopard frog, 13 good morulae were isolated the next day. On the third day, when the fertilized controls were in the gastrula stage, 13 unfertilized punctured eggs were also in the gastrula stage and 4 more eggs were developing abnormally. On the fourth day, 8 of the parthenogenetic eggs had good medullary folds and 4 had irregular folds. On the sixth day, most of the fertilized eggs hatched and 8 of the parthenogenetic eggs hatched also. Of these latter, 4 were developing regularly and 4 irregularly. Those that had not hatched were abnormal.

On the eighth day, the larvae arising from the fertilized eggs were swimming. Among the larvae arising from the unfertilized punctured eggs only 3 were normal, and their development was slightly retarded, perhaps one day. In addition, 6 parthenogenetic larvae were abnormal but still alive.

On the thirteenth day, 2 of the parthenogenetic larvae were feeding and these were the only ones which survived definitely. The other parthenogenetic larvae all died during the next few days. Of the 2 surviving larvae, one went through metamorphosis after five months. When it died, the tail was almost completely absorbed (fig. 1). Its death was probably accidental. The other lived a month longer and formed small hind legs, but died in the tadpole stage (fig. 2).

The sex glands of the frog were taken out, hardened in Tellyesnicki's fluid and sectioned; those of the tadpole were removed after it had been preserved in formalin for several months.

It was found that both parthenogenetic tadpole and frog were females (fig. 3).

This result should be expected if the frog belonged to that group of animals in which the female is heterozygous for sex.

Part of these experiments were made in the laboratory of the University of North Carolina, and we take pleasure in thanking Prof. H. V. Wilson for the many courtesies shown to us.



Figs. 1 and 2 Parthenogenetic frog and parthenogenetic tadpole (natural size)



Fig. 3 Section of the ovary of the parthenogenetic frog (magnification 253)

THE EFFECT OF CONJUGATION IN PARAMECIUM¹

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TWO FIGURES

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¹ Fourth of a series of papers on Heredity, Variation and Evolution in Protozoa.

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I. INTRODUCTION

What is the effect of conjugation on the individual or stock that undergoes it? This question reduces experimentally to the following: In what respect does a stock that has conjugated differ from one that is in other respects similar, but has not conjugated? What difference is produced if half a given stock is allowed to conjugate while the other half is not?

When one examines the evidence for the conclusions commonly drawn as to the effects of conjugation on the stock in the infusoria, it is curious that almost no direct experimental evidence is found. The conclusions as to the rejuvenating or other physiological effects of conjugation are based almost exclusively on reasoning of the following character: "Since without conjugation such and such processes of degeneration (or other phenomena) occur, it must be that conjugation has the effect of preventing or curing this degeneration (or these other phenomena)." The experimentation is almost all devoted to testing the premise, while direct experimental demonstration that the conclusion is correct, that conjugation actually does rejuvenate (or the like), is almost unattempted. Such exceptions to this generalization as exist we shall later take up in detail.

There is then need of an investigation in which conjugation itself—rather than what happens without conjugation—shall lie at the center of experimentation. Such a study this paper pre-

sents. The fundamental experiment is to divide a given stock into two parts, kept under identical conditions, permitting one part to conjugate and preventing the other; to keep these further under identical conditions, and to determine in what respects they differ. In the few attempts that have heretofore been made to observe directly the results of conjugation, the control series (of the same stock *without* conjugation) has almost invariably been omitted, so that it is uncertain how far the phenomena observed would have occurred equally if there had been no conjugation.²

In the investigation here set forth this fundamental experiment has been many times repeated, with careful study of the various characteristics of the set that have conjugated, as compared with those of the set that have not conjugated. Besides an account of the results of this fundamental experiment, the paper deals with certain other problems connected with the physiology of conjugation. The effect of conjugation on the size of the individuals of the stock has been set forth in a former paper (Jennings '11).

Thus the matters dealt with in the present paper are mainly the following: the effects of conjugation on the rate of multiplication; on survival and mortality; on the general vigor; its relation to 'rejuvenescence'; the effects of conjugation among close relatives; the effects of continued inbreeding; the results of allowing a stock to conjugate many times in a given period, as compared with causing it to multiply without conjugation for the same period; the relation of conjugation to inheritance, and the effect of conjugation on variation.

Each experiment gives evidence on most of the matters just mentioned, so that it is not possible to separate fully the different subjects. The experimental results will first be presented systematically, with more particular reference to the effects of conjugation on vigor, multiplication, survival and variation, then each of the topics will be taken up, and an analysis given of the experimental evidence bearing upon it.

² The only exception to this that I have found is in the experiment of R. Hertwig, briefly set forth in his paper of 1889; this will be taken up later.

II. METHODS

Most of the experiments followed a general plan, the chief features of which may be here set forth. The object was, to compare, under similar conditions, a set of the animals that had conjugated with another set that was ready to conjugate, but was prevented from doing so. Both *Paramecium caudatum* and *Paramecium aurelia* were employed in the work. Abundant conjugation was obtained in the way described by Maupas ('89), Calkins and Cull ('07), and others. In the evening large numbers of the animals were taken from the large cultures and placed in watch glasses; early the following morning they were usually beginning conjugation. *Paramecium caudatum* is especially favorable for obtaining with certainty the first stages of the process, since as Maupas ('89, pp. 171, 182) has noted, this animal conjugates in the early morning, commencing at about five o'clock. If therefore there were no conjugations when the watch glasses were set, one can be certain that any pairs found early the following morning have just united.

Split pairs. At the beginning the pairing animals fit loosely together; they at first, as a rule, adhere together only by their anterior ends. At such a time it is easy to separate them, by drawing them repeatedly into a fine pipette. The separated individuals are then isolated and cultivated separately.

Pairs. Other pairs are allowed to complete conjugation. They separate spontaneously after about twelve hours; the two members are then isolated and cultivated separately, under the same conditions as the members of the 'split pairs.'

In this way two sets are obtained, taken from the same culture, both ready to conjugate and beginning the process at the same time; the only difference between them lies in the fact that one is allowed to complete the process, while the other is not. By cultivating the two sets under identical conditions it becomes possible to determine what difference is made by conjugation.

Designation. The terms 'pairs' and 'split pairs' will be used in referring to the members of the two sets, and to their progeny. The two members of any pair, or of any split pair, will be desig-

nated *a* and *b*. It is important to understand that these designations do not imply any characteristic differences, the two letters being assigned arbitrarily and at random to the two members, in order to make it possible to speak of them separately. But the individual and its progeny to which a given letter is assigned of course retain this designation throughout. The pairs (and split pairs) of any experiment are designated by serial numbers, so that any individual is indicated by a number and a letter; thus *8b* signifies the individual *b* of pair 8. The lines of progeny from a given individual receive the same designation as the parent individual, so that in later stages of the experiment *8b* signifies the line of progeny derived from the individual *b* of the pair 8.

Culture. The isolated individuals were transferred to the concavities of hollow ground glass slides, each concavity containing two or three drops of culture fluid. Thick slides with two concavities were found most convenient. At the beginning the animals were usually left for one or two days in water from the dish in which they were found, in order not to disturb the processes of conjugation by the shock of removal to a different fluid. For the later cultivation an infusion of pure Timothy hay was usually employed. This was made by boiling one gram of Timothy hay for ten minutes in 100 cc. of tap water, then adding to this infusion, after it was cool, 100 cc. of filtered but unboiled water. Sometimes this filtered water was taken from the parent culture; a procedure that, in some cases, though not in all, works well. The infusion was tried in varying strengths at different times, but all the animals of a given experiment were treated throughout in exactly the same way. The infusion was invariably made up fresh just before it was used.

In the last experiments tried, it was found that 1% per cent Horlick's malted milk, as recommended by Peebles ('12), was preferable in some respects to the hay infusion, particularly in summer. It was found necessary, for the best results, to make up this culture fluid fresh each day.

The animals were transferred to two drops of fresh infusion, on a clean slide; in some experiments every day, in others every

other day. In some experiments two individuals of each line were transferred to the new slide, in others only one; the remainder being destroyed.

The slides were kept in moist chambers, on strips of glass which were supported above water covering the bottom of the vessel.

Records. At each transfer the number of fissions undergone since the last transfer was recorded; so that these records were made either every day or every other day. Since either one or two individuals had been left on the slide at the previous transfer, there was no difficulty in determining how many fissions had occurred. In some cases of course the same number of fissions had not occurred in the two individuals left the day before, but this made absolutely no difficulty in practice. If, for example, ten individuals were found on the slide, invariably four of these were distinctly smaller than the other six. This showed that each of the two original individuals had divided twice, producing 8, and that two of these 8 individuals had divided again, giving the four small specimens out of the ten. If therefore two of these small individuals were transferred to the new slide (as would usually be done in such a case), the number of fissions was recorded as three.

For keeping records of large numbers of cultures (the numbers ran up to 480 in some cases), the following procedure is convenient. Procure hollow ground slides of which the upper surface has been ground, so that one can write on them with a lead pencil. Then at transfer write upon the new slide (besides the designation of the line) the number of specimens found in the old slide, the number left in the new one, and an indication of the generation to which they belong. Thus in the case just cited, where 10 individuals were present and two of the smaller ones were transferred, the legend on the new slide would be simply '10 (16)-2'; which indicates that 10 were present; that 2 were retained, and that if the fission from which these two resulted were complete, there would have been 16 on the slide. After all the slides have been thus transferred, labeled, and placed in

the moist chambers, the latter are examined and the records on the slides copied to permanent records on large sheets of paper. From these records the exact number of fissions can be obtained at any time; thus, since in the above case the generation containing 16 had been obtained from 2, it is clear that three fissions had occurred.

Tabulation and tables. For analysis, the records of fissions have in most cases been tabulated for definite periods, as of one week or of ten days. In analyzing the records it has been necessary to make from the original tables of records a very large number of secondary tables, particularly correlation tables. In place of publishing these secondary tables, the original tables of record will be published in the Appendix of the present paper. These contain all that would be found in the correlation and other secondary tables, and anyone who desires can reconstruct the latter from them, so that they furnish every possibility for testing the results here given. Furthermore, the original record tables show much that is lost when they are transformed into correlation tables; particularly do they show much that is of interest from the point of view of 'pure line' studies.

Besides these tables giving the original records, the present paper will contain as a rule only tables giving the data—the constants, et cetera—resulting from the analysis, by biometrical methods, of these records; these are found in the body of the paper.

III. EFFECT OF CONJUGATION ON MULTIPLICATION, SURVIVAL AND VARIATION

As previously noted, the experiments described below furnish evidence on other matters besides those set forth in the heading above, but we shall examine them first from this point of view. We shall divide the experiments into two sets; the first including those dealing with 'wild' cultures; the second those dealing with pure strains.

EXPERIMENTS WITH WILD CULTURES: *PARAMECIUM CAUDATUM**Experiment 1: May 4 to June 7, 1909*

As giving typical results, an experiment which was in progress from May 4 to June 7, 1909, will first be presented.

The animals were taken from a 'wild' culture of *Paramecium caudatum*, which was brought from a pool on May 3, 1909. It was found to swarm with the infusoria, and on the evening of May 3 numbers of them were placed in watch glasses; at that time none were conjugating. Early the next morning conjugation was beginning. Thirty-five pairs in the first stages of union were separated in the way already described, but in 11 of these one of the members was killed or lost, so that there remained 59 individuals that had gone through the first stages of pairing; among these 59, both members were present in 24 of the 'split pairs.'

Thirty-one pairs were allowed to complete conjugation, then the two members isolated. One member of one pair was lost, so that from the pairs there were derived 61 lines of propagation, as against 59 from the split pairs.

In this experiment the 120 lines of propagation were changed, and the records taken, every other day. The numbers of fissions were grouped in weekly periods for each line.

Thus we have before us 120 individuals undergoing propagation; one set of 61 have just conjugated, while another set of 59 were ready to conjugate, but were prevented from doing so. They differ in no other way but in regard to conjugation. What later difference does this make in the two sets?

1. The first thing that we discover is that the individuals which were ready to conjugate but were prevented, are by no means in a depressed, degenerate condition, unable to propagate farther. On the contrary, they continue to propagate in an active, healthy manner. They continued to do this till the experiment was discontinued five weeks later.

2. Secondly, we notice that those which have conjugated multiply less rapidly than those which have not. This difference is

very great, and will be well brought out by examining side by side certain weekly records for the first fifteen individuals of each set (table 1).

TABLE I

Experiment 1. Paramecium caudatum. Number of fissions per week for the first 15 lines of each set (d = dead).

First week.															
Pairs	0	1	5	5	4	2	1	5	0	5	6	3	2	4	0
Split pairs	6	6	8	7	7	7	7	7	8	7	8	7	7	7	7
Second week:															
Pairs	1	2	6	5	6	6	0	5	2	4	5	5	1	2	6
Split pairs	4	6	6	7	6	5	4	6	6	4	5	6	5	6	6
Third week:															
Pairs	d	d	8	6	8	1	0	5	0	6	6	3	3	d	8
Split pairs	7	9	6	9	7	6	6	8	6	5	9	10	9	8	7

3. All those which have not conjugated multiply, while among those that have conjugated are a considerable number that either never divide again, although they may live for a long time; or divide but few times. This will be evident from examination of the general record table (table 29, Appendix).

4. A considerable number of the lines derived from those that have conjugated die out, while none of the others die out (table 29).

5. It is evident on a cursory examination of the records that among the lines derived from the conjugants there is much greater variation in the rate of fission than among those derived from the individuals that have not conjugated.

Each of these points will now be taken up in detail and the facts precisely brought out.

The weekly records for the entire experiment are given in table 29 (Appendix), which serves as a basis for the following discussion.

Fifth week not typical. One point should be brought out at the beginning of the analysis. During the fifth week the lines of propagation were in an unhealthy condition owing to extraneous reasons. On May 31, at the end of the fourth week, the experiment was tried of mixing a little starch from boiled bread

with the culture fluid, in the hope that this would improve the latter. It had the reverse effect, making the animals unhealthy, and almost or quite stopping multiplication. As a result, the figures for the fifth week are very low and irregular for both sets. It would beyond doubt give a more correct idea of the real relations if we should exclude the fifth week entirely, and consider the experiment as ending with the fourth week. But I have not felt justified in suppressing any part of the record, and the main results are clear in spite of the irregularity due to the exceptional conditions of the fifth week. But it will be well to keep in mind the fact that the results obtained from the fifth week have little or no significance on our main problems.

Rate of fission. Table 2 gives the mean number of fissions for each of the two sets, for each week and for various combinations, of weeks; also the ratio of the means for those that have not conjugated (split pairs) to the means for those that have conjugated.

TABLE 2

Experiment 1. Mean numbers of fissions per week and for certain other periods, in those that have conjugated (pairs) as compared with the same for those that have not conjugated (split pairs); also ratio of the means for the two sets.

	MEMBERS OF PAIRS		MEMBERS OF SPLIT PAIRS		RATIO OF MEAN FOR SPLIT PAIRS TO MEAN FOR PAIRS
	No.	Mean	No.	Mean	
First week.....	61	3.279 \pm 0.148	59	6.729 \pm 0.099	2.052
Second week.....	56	4.661 \pm 0.205	59	5.932 \pm 0.093	1.273
Third week.....	45	5.000 \pm 0.264	59	6.678 \pm 0.199	1.336
Fourth week.....	42	3.976 \pm 0.210	59	5.102 \pm 0.130	1.283
Fifth week.....	38	2.737 \pm 0.156	59	2.593 \pm 0.113	0.947
First two weeks.....	56	7.589 \pm 0.363	59	12.661 \pm 0.144	1.668
Second two weeks.....	42	9.306 \pm 0.415	59	11.780 \pm 0.287	1.266
Four weeks:					
(a) Those that lived through.....	42	18.857 \pm 0.526	59	24.441 \pm 0.352	1.296
(b) All, including those that did not live to end.....	61	13.918 \pm 0.775	59	24.441 \pm 0.352	1.756
Five weeks:					
(a) Those that lived through.....	38	21.842 \pm 0.602	59	27.034 \pm 0.384	1.238
(b) All.....	61	15.902 \pm 0.833	59	27.034 \pm 0.384	1.700

Table 2 shows that in every week (save the fifth), and in every combination of weeks, the average number of fissions was greater for those that had not conjugated than for those that had conjugated. The fifth week, as we have already seen, gives, for extrinsic reasons, atypical results. In that particular case the difference in the means is not significant, as is shown by the probable errors in the two cases. For the entire four (or five) weeks, the average number of fissions was about 25 per cent greater in those that have not conjugated. If we take the total number of fissions for each line that was alive at the beginning of the experiment, we find that the average number of fissions was 70 to 75 per cent greater for those that had not conjugated. This is of course partly due to the fact that none of the latter died before the end of the experiment, while a considerable number of the conjugant lines died out early.

It is of interest to compare the number of progeny produced by the two sets. This is of course obtainable from the number of fissions. The potential progeny produced by the two sets in each of the five weeks is given in table 3.

As the table shows, each line of those that have not conjugated produced weekly on the average almost exactly two-and-a-half times as many progeny as a line of the conjugants.

The 61 lines derived from the conjugants had a potential production all together during four weeks of the experiment of

TABLE 3

Experiment 1. Potential number of progeny from those that have conjugated, as compared with those that have not, based on the number of fissions in table 2.

WEEK	PAIRS			SPLIT PAIRS		
	Number of lines	Number of progeny	Average per line	Number of lines	Number of progeny	Average per line
1	61	991	16.246	59	8008	135.729
2	56	3038	54.250	59	4296	72.813
3	45	4712	104.711	59	17066	289.254
4	42	1489	35.452	59	2962	50.203
5	38	479	12.605	59	537	9.102
Average per week.....			44.653	111.429		

1 billion, 256 million progeny, while the 59 lines derived from those that had not conjugated had a production of 48 billion, 467 million, so that the non-conjugants produced somewhat more than 38 times as many progeny as the conjugants. The very great difference between the two in this respect arises from the fact that many of the conjugant lines died out before the end of the experiment and the further fact that the number of progeny increases in geometrical ratio as the number of fissions increases in arithmetical ratio. To this latter fact is due also the seemingly excessive differences in the number of progeny produced in the different weeks, as shown in table 3. Unfavorable temperature or culture medium, decreasing the number of fissions by a small number, decreases the progeny enormously.

To sum up on this point, the experiment shows clearly that those that have not conjugated multiply more rapidly than those that have conjugated, and the difference persists for at least four weeks after conjugation.

Variation. A careful examination of the data given in table 29 will show that there is more variation (among the different lines) for the number of fissions in any given period, for those that have conjugated than for those that have not. To determine accurately the differences in this respect, it is necessary to determine the standard deviations and coefficients of variation for each period. These are given in table 4 together with a comparison showing what the ratio of the variation among the non-conjugants is to that among the conjugants.

Table 4 shows that in every week, and in every combination of weeks, without exception, the variation is greater in those that have conjugated than in those that have not. It is greater in the conjugants, whether measured absolutely, by the standard deviation; or relatively to the mean, by the coefficient of variation. In many of the periods the coefficient of variation is for the non-conjugants but one-third to one-fourth of that for the conjugants.

It is then a simple statement of fact to say that in this case conjugation increased greatly the variability in the fission rate. Examination of table 29 shows that this great increase of varia-

tion in the progeny of the conjugants is due mainly to the fact that many of the lines descended from them multiply but slowly, while others multiply at nearly the same rate as do the progeny of non-conjugants. Among the 59 lines of non-conjugants, there are but two that gave fewer than 20 fissions in the five weeks, while among the 38 lines of conjugants that lived through the entire five weeks there are 12 that fall below 20. On the other

TABLE 4

Experiment 1. Relative variability in number of fissions for given periods, in those that have conjugated (pairs), and those that have not (split pairs).

	PAIRS			SPLIT PAIRS			RATIO OF SPLIT PAIR TO PAIR
	No.	Standard deviation	Coefficient of variation	No.	Standard deviation	Coefficient of variation	
First week.....	61	1.709 \pm 0.104	52.131 \pm 3.955	59	1.132 \pm 0.070	16.829 \pm 1.074	0.662 0.323
Second week.....	56	2.270 \pm 0.145	48.704 \pm 3.769	59	1.055 \pm 0.066	17.792 \pm 1.139	0.465 0.365
Third week.....	45	2.625 \pm 0.189	52.494 \pm 4.648	59	2.266 \pm 0.141	33.929 \pm 2.337	0.863 0.616
Fourth week.....	42	2.018 \pm 0.149	50.743 \pm 4.596	59	1.481 \pm 0.092	29.027 \pm 1.948	0.734 0.572
Fifth week.....	38	1.427 \pm 0.110	52.135 \pm 5.011	59	1.290 \pm 0.080	49.750 \pm 3.778	0.904 0.954
First two weeks	56	4.030 \pm 0.257	53.103 \pm 4.232	59	1.643 \pm 0.102	12.975 \pm 0.819	0.408 0.214
Second two weeks.....	42	3.991 \pm 0.294	42.870 \pm 3.689	59	3.268 \pm 0.203	27.743 \pm 1.850	0.816 0.647
Four weeks:							
(a) Those that lived through	42	5.055 \pm 0.372	26.806 \pm 2.110	59	4.010 \pm 0.249	16.405 \pm 1.046	0.793 0.612
(b) All.....	61	8.901 \pm 0.544	63.951 \pm 5.430	59	4.010 \pm 0.249	16.405 \pm 1.046	0.451 0.257
Five weeks:							
(a) Lived through	38	5.469 \pm 0.425	25.174 \pm 2.068	59	4.376 \pm 0.272	16.188 \pm 1.031	0.796 0.643
(b) All.....	61	9.571 \pm 0.585	60.186 \pm 4.829	59	4.376 \pm 0.272	16.188 \pm 1.031	0.457 0.269

TABLE 5

Experiment 1. Paramecium caudatum. Number of lines that died out during different periods, among those descended from the pairs (conjugation consummated).

	WEEK					FOUR WEEKS	FIVE WEEKS
	1	2	3	4	5		
Number died.....	0	5	11	3	4	19	23
Per cent of those alive at beginning of week.....	0	8.2	19.6	6.7	9.5		
Per cent of all.....	0	8.2	18.0	4.9	6.6	31.15	37.7

hand, the upper extreme for the non-conjugants (38) is higher than that for the conjugants (32), but most of the non-conjugants are so grouped near the high figure that the variation is relatively small.

Mortality. None of the 59 lines of non-conjugants died out during the five weeks of the experiment. Of the 61 lines of conjugants, on the other hand, 23, or 37.7 per cent, died out during the experiment. The number of lines descended from conjugants that died out during each week is given in table 5.

Thus in this case conjugation greatly increased the mortality. Although the 'split pairs' were ready to conjugate, and had actually taken the first steps in the process, they are not in the least injured by being prevented from consummating the process; while those that finished mating showed a high mortality.

Abnormalities. Besides the actual deaths, the descendants of those that had conjugated showed many abnormalities, while among the descendants of the non-conjugants there were none. For example, on May 14, I noted that there were among the descendants of the conjugants 24 abnormal individuals, belonging to 12 different lines, while in the other set there were none.

The abnormalities take the most diverse forms: bodies of irregular shape, crooked, truncate, or with projections; double or multiple monsters: some are abnormally large, others extremely thin. The structural abnormalities are in many cases connected with abnormalities in fission. In some cases the ex-conjugants do not divide for many days after separation. During this time they grow larger till they reach an immense size, many times greater than that ever reached at other times. Some of these immensely large individuals never divide again, and after living a week or two die. Others after a time divide irregularly, producing progeny of diverse sizes and forms. Thus the individual 10a, of the pairs, in this experiment did not divide until eight days after the separation from its mate. It then divided during the night into seven, of four diverse sizes. The individual 17b divided immediately into two specimens, which became immensely large; these did not divide again for six days, then each produced two large abnormal individuals which soon died.

Abnormal individuals appear again and again in certain of the lines derived from conjugants, while in others they do not appear at all. The conditions which induce them are thus evidently inherited from generation to generation in the fissions. As a rule, a given abnormality is not inherited in its special form, but only the tendency to produce abnormalities of various sorts.

Lines which show abnormalities in structure commonly have a slow rate of fission, are thin, succumb easily to unfavorable conditions, and in general, appear to lack vitality. Often they die out after a number of generations.

There are likewise found lines which show the thinness, slow fission rate, and general lack of vitality, without structural abnormalities.

It appears probable that these abnormalities have a cytological basis, and are due to irregularities in the nuclear processes accompanying conjugation. A precise study is greatly needed, as to the minute characteristics of these abnormalities, their heritability, their experimental cause, and their cytological basis. Such a study I hope will soon be made.

The data obtained from this experiment, and presented in table 29, will be analyzed in later papers with reference to the problems of sexuality, and of uniparental and biparental inheritance.

We may summarize the results of this experiment, so far as they bear on the problems now under consideration, as follows:

Conjugation decreases the rate of fission, causes a great increase in variation in the fission rate, brings about many abnormalities, and greatly increases the death rate.

Experiment 2: April 7 to June 7, 1909

This extensive and long continued experiment was the first one undertaken for comparing the fission-rate and vitality of animals that had conjugated and animals that had not. Owing to lack of experience the method of culture was not good, so that the mortality was very high; this makes the results less sharp and clear than in the experiment just described. The chief mistakes in culture were: (1) the culture fluid was not made up

by measure, so that it varied in strength from day to day. All the specimens were treated alike at each change, so that no difference between the sets resulted from this; but the changes in concentration of the infusion caused many deaths. (2) The hay of which the infusion was made was not sorted over, to exclude all but Timothy; thus at times injurious plants were included, increasing the mortality. (3) As a rule, only one individual of each line was retained at each change, so that if this individual died, the line became extinct. As a consequence of all these things, the number of lines decreased rapidly in all the different sets.

However, such an experiment, lasting eight weeks, is not likely to be often repeated, and the results are of much value in certain relations.

Three sets of the animals were employed in this experiment. One set ('pairs') consisted of individuals that had just conjugated; a second ('split pairs') included individuals that had begun to unite for conjugation, but were separated, in the manner previously described; the third set ('free') consisted of ordinary individuals that had not begun union, taken from the same culture as the others. This third set is known not to have conjugated recently, since they were taken (like the others) in the early morning, from watch glasses which contained no conjugants when set the evening before. Comparison of the 'split pairs' and the 'free' will show whether entrance upon the condition preparatory to conjugation alters the animals in any way, such as to affect their multiplication and vigor.

The 'free' specimens were given paired designations, each two being called *a* and *b*, as in the 'pairs' and 'split pairs'. In this case of course *a* and *b* were related in no way; the paired designations were given at random, in order to test the question whether one individual of a pair of conjugants dies or is weak more often than occurs as a result of mere chance causes, in specimens paired at random and merely by designation. This is a matter that will be dealt with fully in a later paper.

The three sets were treated in exactly the same way, the slides of pairs, split pairs and free alternating in the same moist cham-

bers. The culture fluid was changed as a rule every other day.

Since usually members of pairs of conjugants do not divide till the second day after conjugation, the comparison of the rate of fission for the three sets was not begun till this second day. Thus, the animals were isolated on the morning of April 8, but the tabulation of the fissions begins, for all sets, on April 10.

For purposes of comparison, the fissions were tabulated by weeks for each of the three sets. The experiment may best be divided into two periods, the first comprising the first two weeks; the second the last six weeks. At the end of the second week a considerable number of each set were lost by accident, so that the number to be dealt with is much smaller in the second period.

The experiment included at the beginning 57 lines ($28\frac{1}{2}$ pairs) of those that had finished conjugation; 39 lines ($19\frac{1}{2}$ pairs) of split pairs, and 58 lines of 'free' individuals.

The actual number of fissions per week is given for the first two weeks in table 30; for the last six weeks in table 31 (Appendix).

It should be noted that the data given are, so far as numbers of fissions go, of little value after the sixth week, and particularly is this the case for the seventh week. Pressure of other duties forced me to neglect these experiments at that time, so that during the seventh week the slides were changed but once; as a result they hardly multiplied at all. The figures for the seventh and eighth weeks are given only in order not to suppress any part of the record.

It is evident, as in the previous experiment, that the animals which were ready for conjugation were by no means in a depressed or degenerate condition. The split pairs continue to multiply, somewhat more rapidly than those that have conjugated. We shall examine in detail the rate of fission, the variation, and the mortality, in the three sets.

Rate of fission. Table 6 gives the mean numbers of fissions in each set, for each week, and for certain other periods.

As this table shows, in practically all of the 15 means given, the rate of fission is less for those that have conjugated than for those that have not. The only exception is in the seventh

week, where the rate is nearly the same, with a slight excess in favor of the conjugants. But as we have already noted, the animals were changed and (records made) but once that week, and in consequence there was almost no multiplication; the figures for that week are of no significance. In all the other cases (14 out of 15) the non-conjugants show a greater rate of fission, the excess varying from 6 to 85 per cent, with an average of 23 to 31 per cent.

The split pairs and the free individuals show no significant difference so far as rate of fission is concerned; so that the specimens that have taken the first steps in conjugation do not differ in this respect from those that have not.

TABLE 6

Experiment 2. Mean number of fissions for each week, and for certain other periods, in the three sets, together with a comparison of all that have conjugated with all that have not.

WEEK	PAIRS		SPLIT PAIRS		FREE		ALL NON-CONJUGANTS SPLIT + FREE		COMPARISON OF CONJUGANTS AND NON-CONJUGANTS	
	Number of lines	1	Number of lines	2	Number of lines	3	Number of lines	4	Excess of non-conjugate over that of conjugants	Per cent of excess in terms of conjugant mean
		Mean number of fissions		Mean number of fissions		Mean number of fissions		Mean number of fissions		
1	50	4.080 ± 0.150	37	7.378 ± 0.162	54	7.685 ± 0.121	31	7.560 ± 0.102	3.480	85.3
2	34	2.000 ± 0.126	21	2.810 ± 0.174	30	2.233 ± 0.126	51	2.471 ± 0.106	0.471	23.6
3	22	3.591 ± 0.236	10	4.800 ± 0.230	19	4.947 ± 0.197	29	4.867 ± 0.160	1.306	36.4
4	19	3.211 ± 0.335	9	4.666 ± 0.237	17	4.471 ± 0.178	28	4.538 ± 0.241	1.327	41.3
5	14	7.143 ± 0.609	8	9.125 ± 0.186	15	9.333 ± 0.164	23	9.261 ± 0.129	2.118	29.7
6	11	5.182 ± 0.272	8	5.125 ± 0.512	14	5.714 ± 0.159	22	5.500 ± 0.215	0.318	6.1
7	11	1.455 ± 0.133	8	1.125 ± 0.186	14	1.429 ± 0.089	22	1.318 ± 0.091	-0.137	-9.4
8	9	4.111 ± 0.430	6	5.333 ± 0.130	11	4.636 ± 0.264	17	4.882 ± 0.185	0.771	18.8
1 and 2	34	6.176 ± 0.229	21	10.381 ± 0.296	30	10.100 ± 0.281	51	10.216 ± 0.206	4.040	65.4
3 and 4	19	7.158 ± 0.520					26	11.692 ± 0.253	4.534	63.3
5 and 6	11	13.727 ± 0.451					22	14.773 ± 0.267	1.046	7.6
6 weeks	11	29.364 ± 0.645	8	35.000 ± 0.860	14	35.143 ± 0.486	22	35.091 ± 0.440	5.727	19.5
8 weeks	9	34.111 ± 0.962	6	42.500 ± 0.910	11	41.545 ± 0.645	17	41.882 ± 0.532	7.771	22.8
Mean of Mean per week for those that survived weeks	9	4.264	6	5.313	11	5.193	17	5.235	0.971	22.8
		3.846		5.045		5.056		5.053	1.207	31.4

TABLE 7

Experiment 2. Relative variability in fission rate for those that have conjugated and those that have not.

WEEK	CONJUGANTS ('PAIRS')			NON-CONJUGANTS ('SPLIT PAIRS') AND ('FREE')		
	No.	Standard deviation	Coefficient of variation	No.	Standard deviation	Coefficient of variation
1	50	1.573 ± 0.106	38.549 ± 2.962	91	1.447 ± 0.072	19.434 ± 0.991
2	34	1.085 ± 0.088	54.235 ± 5.590	51	1.126 ± 0.075	45.592 ± 3.623
1 + 2	34	1.977 ± 0.162	32.011 ± 2.874	51	2.181 ± 0.146	21.350 ± 1.489
3 + 4	19	3.360 ± 0.368	46.944 ± 6.166	26	2.671 ± 0.250	22.847 ± 2.246
5 + 6	11	2.219 ± 0.319	16.168 ± 2.385	22	1.857 ± 0.189	12.570 ± 1.298
6 weeks...	11	3.170 ± 0.456	10.796 ± 1.571	22	3.059 ± 0.311	8.716 ± 0.893
8 weeks...	9	4.280 ± 0.680	12.548 ± 2.026	17	3.252 ± 0.376	7.764 ± 0.904

The split pairs and the free may therefore properly be considered together, as non-conjugants, as in the fourth column of table 6. With these total results for all the individuals that have not conjugated may then be compared the results for those that have conjugated, as in columns 5 and 6 table 6. As there shown, for the first week the excess of the non-conjugants was 85 per cent, so that their rate was nearly double that of the conjugants. After this the excess for the non-conjugants decreased, although even in the eighth week it is 18.8 per cent. Thus the conjugants had not regained a rate equal to that of the non-conjugants even after so long a period.

Variation. The variation in the rate of fission is shown comparatively for conjugants and non-conjugants in table 7. In this table the two classes of non-conjugants—the 'split pairs' and the 'free'—have been put together, since we have already seen from table 6 that there is no characteristic difference between them. This fact is shown equally if we compute the variation separately for the two classes. Thus, for the first week the split pairs give a standard deviation of 1.458 and a coefficient of 19.767, while the corresponding figures for the free are 1.425 and 18.542. For the second week the figures are: split pairs, 1.180 and 41.998; free, 1.023 and 45.785; first two weeks, split pairs, 2.011 and 19.375; free, 2.285 and 22.628. Throwing the two together, as in table 7, gives the great advantage of larger numbers.

As the table shows, although the means for the conjugants are throughout less (table 6), their standard deviations are as a rule greater than the standard deviations for those that have not conjugated. As a result, the coefficient of variation (standard deviation divided by the mean) is in every case much greater for those that have conjugated. For the first week the variation, as measured by this coefficient, is twice as great in the conjugants. For the entire eight weeks it is nearly twice as great.

Thus in this experiment, as in the former one, conjugation has the effect of greatly increasing the variability of the fission rate.

Mortality. Owing to the high general mortality, due to imperfect culture methods, the distribution of deaths in this experiment is of much less significance than in Experiment 1. It is summarized in table 8.

As the table shows, the death rate was greater in those that had conjugated than in those that had not, in every week save the second. In the second week I tried the experiment of adding to the cultures water from a pool that was extremely foul, but contained many *Paramecia*. It proved disastrous; many of my lines were killed, and among these were a larger proportion of the split pairs and free than of the pairs. I doubt if the distribution in such a catastrophe is of any significance; though possibly it indicates that those that have conjugated are more resistant to such decidedly injurious conditions.

Throughout the remainder of the experiment (as throughout the entire time in Experiment 1), the mortality was highest among those that had not conjugated. For the entire eight weeks together the mortality is nearly the same for all three classes, but is a little greater for those that have conjugated.

There appears to be no significant difference, as to mortality, between the split pairs and the free. There is thus no indication that prevention of a conjugation that had been initiated is in any way injurious.

Abnormalities. In this experiment, as in Experiment 1, I noted frequent abnormalities among the progeny of the conjugants; none among the other sets. No detailed study was made of these.

TABLE 8

Experiment 2. Death rate in pairs, split pairs and free individuals.

	NUMBER AT BEGINNING	DIED	PERCENT DIED		NUMBER AT BEGINNING	DIED	PERCENT DIED
First week:				First two weeks			
Pairs.....	57	7	12.3	Pairs.....	57	21	36.8
Split pairs.....	39	2	5.1	Split pairs.....	39	18	46.2
Free.....	58	4	6.9	Free.....	58	28	48.3
Second week				Last six weeks			
Pairs.....	50	14	28.0	Pairs.....	28	19	67.9
Split pairs.....	37	16	43.2	Split pairs.....	13	7	53.8
Free.....	54	24	44.4	Free.....	23	12	52.2
Third week:				Last five weeks			
Pairs.....	28	6	21.4	Pairs.....	22	13	59.1
Split pairs.....	13	3	16.7	Split pairs.....	10	4	40.0
Free.....	23	3	13.0	Free.....	19	8	42.1
Fourth week				Total eight weeks			
Pairs.....	22	3	13.6	Pairs.....	49	40	81.6
Split pairs.....	10	1	10.00	Split pairs.....	31	25	80.6
Free.....	19	2	10.5	Free.....	49	38	77.6
Fifth week:							
Pairs.....	19	5	26.3				
Split pairs.....	9	1	11.1				
Free.....	17	2	11.1				
Sixth week:							
Pairs.....	14	3	21.4				
Split pairs.....	8	0	0				
Free.....	15	1	6.7				
Seventh week:							
Pairs.....	11	0					
Split pairs.....	8	0					
Free.....	14	0					
Eighth week:							
Pairs.....	11	3	27.3				
Split pairs.....	8	2	25.0				
Free.....	14	3	21.4				

¹ Eight of the pairs, 8 of the split pairs, and 9 of the free, were accidentally lost during the experiment, so that their disappearance is not accounted for in this table.

Summary of results. Experiment 2 gives the following general results:

In three sets of individuals taken from the same culture and treated in the same way, one set that was allowed to complete conjugation, another separated before union was complete, and a third that had not yet begun conjugation:

1. Those that had completed multiplied throughout less rapidly than those that did not complete conjugation, and less rapidly than those that had not begun conjugation. This difference persisted throughout the experiment; that is, for eight weeks after conjugation had occurred.

2. The descendants of those that had completed conjugation were much more variable in their rate of fission than those that did not conjugate.

3. The mortality was slightly greater among those that had completed conjugation than among the others.

4. There was no marked difference in these respects between the set that were separated after beginning conjugation, and the set that had not yet begun.

Experiment 3: June 20 to June 24, 1909: Effect of high temperatures

This experiment lasted but four days, and was designed primarily to test the relative variability in the dimensions of the progeny of conjugants and of non-conjugants. The results on this point have been given in my paper of 1911, on Assortative mating, et cetera (table 32, p. 99). Here will be given the results of the experiment so far as they bear upon the comparative vitality and the rate of reproduction in conjugants and non-conjugants.

In the experiments which we have thus far described, the conjugants reproduced more slowly than the non-conjugants, while at the same time the mortality of the conjugants was higher. It appears possible that under some conditions the greater rapidity of fission of the non-conjugants might be disadvantageous, causing greater mortality in them than in the conjugants. This possibility is realized in the present experi-

TABLE 9

Experiment 3. Paramecium caudatum. Relative number of fissions for the conjugants and non-conjugants: during the four days, June 20 to June 24, 1909. (Including only those that lived throughout the four days.)

	NUMBER OF FISSIONS													TOTAL	MEAN
	2	3	4	5	6	7	8	9	10	11	12	13			
Number of conjugant lines	1	1	4	7	8	6	4	4	1				36	6.222	
Number of non-conjugant lines							1	2	3	5	3	2	16	10.813	

ment. The temperature during the four days that the experiment lasted was excessively high, the thermometer standing much of the time above 90° F. (above 32°C.). The non-conjugants multiplied with furious rapidity, at the rate of two to four fissions a day (one fission in 6 to 12 hours), so that the average for all that lived through was a little over two and a half per day (one fission in 9½ hours). The conjugants, on the other hand, multiplied much less rapidly, the rate being but one-and-a-half per day, or one fission in eighteen hours.

Correlative with this excessively rapid rate of reproduction, the non-conjugants showed a very high mortality. At the end of four days, 35 of the original 51 lines were dead, so that the mortality was 68.6 per cent. In the conjugants, on the other hand, of the original 47 lines, only 11 died, or but 23.4 per cent.

The data for the rate of fission of the conjugants and non-conjugants in this experiment are given in table 9.

The results of this experiment agree with those of all the others in showing that conjugation decreases the rate of fission. They differ from those of all others in the fact that the mortality is much greater in the non-conjugants. The result, due, as it evidently is, to the excessive rate of fission induced in the non-conjugants by the very high temperature, shows that under certain conditions conjugation may have a directly protective effect, owing to its decreasing the rate of multiplication. Interesting results would be obtained by comparing conjugants and non-conjugants of the same stock under diverse conditions; high and low temperatures, different chemical conditions, et

cetera. Possibly it would be found that under all conditions tending to cause excessive rapidity of fission, conjugation is protective by decreasing this rate.

The usual relations are found as to the relative variability of the conjugants and non-conjugants. In my paper on Assortative mating ('11, p. 99), I have shown that the progeny of the conjugants are in this experiment much more variable in size than the progeny of the non-conjugants, for at least seven generations. Here we need to consider only the variability in fission rate.

Of the non-conjugants, as we have seen, but sixteen lived through the four days. Their mean rate of fission is 10.813 ± 0.233 , the standard deviation is 1.379 ± 0.164 , and the coefficient of variation is 12.756 ± 1.546 . Of the conjugant lines, thirty-six lived through; their mean number of fissions was 6.222 ± 0.205 , the standard deviation 1.827 ± 0.145 , and the coefficient of variation 29.369 ± 2.528 . Thus the variation is both absolutely and relatively much greater in the conjugants; if we measure it by the coefficient of variation, the variability in fission rate was more than twice as great in the progeny of the conjugants as in that of the non-conjugants.

EXPERIMENTS ON PURE STRAINS: CONJUGANTS ALL DESCENDED
FROM A SINGLE INDIVIDUAL

A large number of experiments, some of them extensive and long continued, were undertaken with cultures descended from a single individual. The conditions in such pure strains (or 'pure lines', as I have called them in previous papers), are of special interest in some respects, while the results bear likewise upon the same general problems as does the work with wild cultures.

Race k. These experiments were mostly carried on with the race *k*, some account of which has been given in my previous papers of 1910 and 1911. This race, belonging to the species *Paramecium aurelia*, is distinguished by a tendency to conjugate frequently, making it most favorable material for a study of

matters connected with conjugation. It is easy to induce epidemics of pairing at intervals of about a month, and they sometimes occur at much shorter intervals. In order to make clear the conditions with which we are dealing, it is necessary to give a brief account of the history of this race.

As a pure strain, the race *k* is derived from a single ex-conjugant isolated November 9, 1908. This individual itself came from a culture derived from eight pairs of conjugants taken February 4, 1908, these eight pairs being themselves derived from 10 single individuals of similar size, taken from a wild culture January 29, 1908. Thus even before the destruction of all but this single individual of November 9, the race *k* was derived from few individuals, which very possibly all came from one. Our first experiment given below (Experiment 4) made use of this race *k* before its absolutely certain derivation from a single individual; all the rest employed *k* as known to be a pure strain.

Epidemics of conjugation were observed in this race eight times between January 29 and its absolute purification on November 9. Since November 9 a great number of conjugations have been observed; records have been kept of at least twenty.

That portion of the race still in existence (July, 1912), and the part on which some of the chief experiments were performed, has descended from many successive conjugations, in which all the surviving progeny were derived from a single member of a pair. Eight such conjugations have been observed, so that all the animals now existing (and used in Experiments 13 and 14, below) are derived from eight generations of the strictest inbreeding—all the members of each of these generations being derived from the fission of a single individual. This inbred race seems healthy and vigorous, so long as cultivated in mass culture. But it appears to have lost the ability, which it had at the beginning, to propagate for any considerable period on slides. On this account it has of late become unavailable for comparative work on such questions as the rate of reproduction and the way this is affected by conjugation or by other conditions; a fact which has caused much trouble and loss of time. Many extensive experiments

have been undertaken, but after two or three weeks of intense labor, all representatives of this race *k* cultivated on the slides have become unhealthy and died. Earlier in its history it was kept on slides for months in succession, multiplying vigorously throughout. Whether its present peculiarity in this respect has any connection with the long continued inbreeding, or whether it may be due only to weakening from previous long cultivation on slides, it is difficult to say; there is some indication, as we shall see, that the latter is the case.

Experiment 4: October 19 to November 8, 1908: Paramecium aurelia

The first experiment on conjugation in race *k* was designed primarily to permit a comparison of the dimensions of the progeny of conjugants and non-conjugants in the same race. The results so far as dimensions are concerned are given in my paper of 1911 on Assortative mating (pp. 96-97). Incidentally, the records kept give data as to the relative rate of fission, and as to mortality. As we have noted above, in this experiment (alone of all those with *k*), the race is not yet known to be absolutely pure, in the sense of derived from a single individual, without admixture from others; it is, however, extremely homogeneous, and probably quite pure, even at this time.

The experiment was begun with 46 paired individuals (23 pairs), and 46 that were non-conjugants, derived from the same culture. During the course of the experiments 4 of the conjugant and 8 of the non-conjugant lines were accidentally lost, leaving 42 of the former and 38 of the latter.

This was one of the earliest experiments of the sort that I tried, and the mortality was very high, doubtless owing to inexperience in handling. Of the 42 lines derived from the conjugants, but 17 lived throughout the twenty days of the experiment, while of the non-conjugants 18 lines lived through. The total number of fissions for each of these 35 surviving lines is given in table 10.

For the 17 conjugant lines the mean number of fissions is 13.294 ± 0.670 , with a standard deviation of 4.098 ± 0.474 and

a coefficient of variation of 30.828 ± 3.890 . For the 18 non-conjugant lines the mean is 13.500 ± 0.425 ; the standard deviation 2.672 ± 0.300 , the coefficient of variation 19.792 ± 2.310 .

Thus here, as in all other cases, the rate of fission is a little greater in the non-conjugants, while the variability is much greater in the progeny of the conjugants than in that of the non-conjugants.

TABLE 10

Experiment 4. Paramecium aurelia. Number of fissions for the conjugants and non-conjugants during the twenty days of experiment 4 (October 19 to November 8, 1908).

	NUMBER OF FISSIONS																	TOTAL	MEAN
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
Number of conjugant lines.....	1						1	1					2	4	3	2	3	17	13.294
Number of non-conjugant lines.....						1		1				1	3	8		2	2	18	13.500

Where the mortality is so high as in this case, it is doubtless due mainly to extrinsic causes, so that its distribution is of little significance. The facts are these: of 42 lines descended from conjugants, 25 died out during the twenty days of the experiment, a mortality of 59.52 per cent. Of the 38 lines descended from non-conjugants, 20 died out, a mortality of 52.63 per cent. Thus the mortality is, as usual, greatest among the descendants of the conjugants.

Experiments 5 to 14: Comparative effects of repeated conjugation, and of long abstention from conjugation

During the year 1910 a very extensive series of experiments was carried on with the pure race *k*, for testing the relative effects of conjugation and of abstention from conjugation. The whole series was so bound together that it might well be considered one prolonged experiment; it will be convenient, however, in giving an account of it, to designate as separate experiments the various phases of it.

Diagram of history of these experiments. We have already (page 302) given some account of the race *k*. To make clear the conditions in the present series of experiments, I give a diagram (fig. 1) showing the history of the various divisions of this race with which we are dealing; reference to this diagram should frequently be made in reading the text. The race *k* as dealt with in these experiments was derived from a single ex-conjugant of November 9, 1908; before these experiments were undertaken, it had passed through three self-fertilizations, or conjugations with inbreeding; that is, all the surviving members of the race were descendants by fission of a single individual of the preceding conjugation; so that the two individuals that make up a pair were thus descended from one. (In a fourth conjugation in the series, on March 9, 1909, 25 pairs were saved, so that all came from these; see diagram, fig. 1.)

After the fourth conjugation, of May 24, 1909, the culture was allowed to rest till January 29, 1910; during the interval there may have been many conjugations, in which of course the individuals would mate at random. On January 29, 1910, a pair was isolated, from a single member of which came the line of cultures which we shall call *B*; it forms the branch designated *B* in our diagram (fig. 1).

In the remainder of this culture a new conjugation occurred March 4, 1910. At this time there were isolated certain ex-conjugants, one of which gives the series forming the branch *C*, one of the three main branches in our diagram; there were also

Fig. 1. Diagram showing the nature and history of Experiments 5 to 14, and the history of the pure strain *k*, employed in this work. The rectangles (bounded by broken lines) indicate each an experiment, and show what sorts of individuals were compared, with the history of each. A united pair indicates the progeny of conjugants; a single individual, the progeny of non-conjugants. Thus, in Experiment 9, there were compared the progeny of non-conjugants (of branch *A*), and of conjugants (branch *B*) that had gone through four conjugations since the others had conjugated. The dates beside each rectangle show the length of time that this experiment lasted. The numbers in or (near) the rectangles give the number under which the experiment is described in the text. The pairs at the left show (with dates) the known self-fertilizations that the race had undergone before these experiments began, the survivors being in each case derived from a single ex-conjugant, save after the conjugation of March 9, 1909.

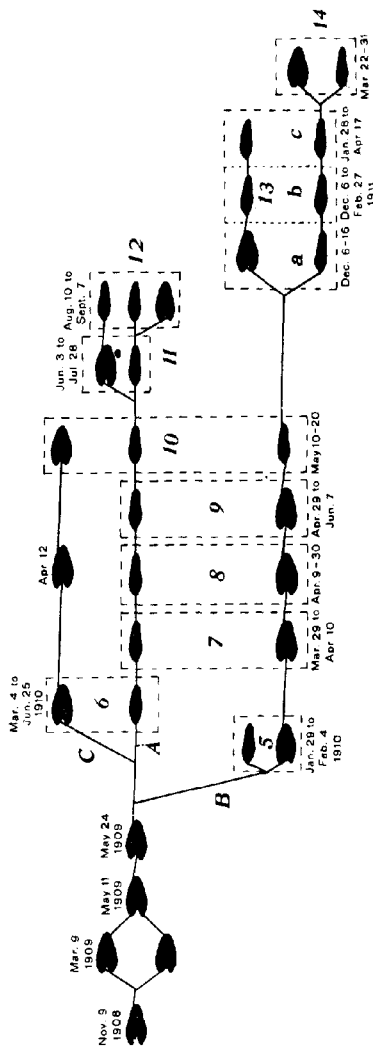


Figure 1

isolated certain split pairs, in which conjugation had not been consummated; one of these gives the long line forming the branch *A* of our diagram.

This branch *A* was then propagated without conjugation, from some period before March 4, 1910, while the other two lines *B* and *C* conjugated repeatedly; the experiments consisted mainly in comparison of the progeny from these conjugations with the progeny of the non-conjugating branch *A*. Finally, on June 4, a part of *A* was allowed to conjugate, the rest not, and these two parts compared. Again, on August 10, a part was allowed to conjugate, and compared with the part that had propagated from the beginning of the experiment without conjugation. In the following an account is given of the results of these various comparisons.

Experiment 5: January 29 to February 4, 1910: Paramecium aurelia

Experiment 5 was a brief one, dealing with 10 pairs (20 individuals) and 10 split pairs (20 individuals), belonging to the branch *B* of race *k* (fig. 1). These 40 lines were cultivated side by side under the same conditions for six days. Of the descendants of the pairs, three were accidentally lost during the course of the experiment, so that we have finally but 17 lines descended from conjugants; 20 descended from non-conjugants.

Of the 17 lines of conjugants, 8 died out during the experiments, a mortality of 47.06 per cent. Of the 20 lines of non-conjugants, 9 died out, a mortality of 45 per cent.

The number of fissions for the six days of the experiment is given for the surviving conjugant and non-conjugant lines in table 11, while the results are summarized in table 12.

Thus, as the tables show, this experiment, so far as it goes, illustrates the usual conditions:

1. Those that have conjugated multiply less rapidly than those that have not.
2. The rate of fission is much more variable among those that have conjugated—both the standard deviation and the coeffi-

TABLE 11

Experiment 4. Paramecium aurelia. Number of fissions for the descendants of pairs and split pairs, during the six days from January 29 to February 4, 1910.

	NUMBER OF FISSIONS							TOTAL	MEAN
	0	1	2	3	4	5	6		
Conjugant lines (pairs).....	1			2	2	3	1	9	3.889
Non-conjugants (split pairs).....					2	3	6	11	5.364

TABLE 12

Experiment 5. Summary of results as to mortality, rate of fission and variability, in conjugant and non-conjugant lines, for the six days of the experiment.

	NUMBER DIED	PER CENT DIED	MEAN FISSIONS	STANDARD DEVI- ATION	COEFFICIENT OF VARIATION
Pairs.....	17/8	47.06	3.889 \pm 0.272	1.663 \pm 0.192	42.762 \pm 5.781
Split pairs.....	20/9	45.00	5.364 \pm 0.116	0.771 \pm 0.082	14.382 \pm 1.565

cient of variation being more than twice as great as for the non-conjugants.

3. The mortality is higher among those that have conjugated.

Experiment 6: March 5 to June 25, 1910: Paramecium aurelia

From March 5 to June 25, 1910, a period of sixteen weeks, a further experiment was carried on with this pure line *k*, giving results as to the difference between those that have conjugated and those that have not. The experiment was primarily a study of the fission rate and its inheritance in different races and under different conditions, so that it included a large number of lines of propagation, of diverse character. Among these were twelve lines from the same culture, six beginning with members of pairs that had just conjugated, and six others derived from individuals that were beginning conjugation, but were separated before the process had been accomplished (split pairs). It is only with the results from these twelve sets, bearing on the effects of conjugation, that we shall deal here, reserving the remainder of the experiment for a paper dealing with the inheritance of the rate of fission.

A watch glass culture of the race *k* showed on March 4 the beginnings of conjugation, many of the individuals being observed in the act of uniting; (for the history of the race before and after this time, see the diagram, fig. 1). Three pairs were separated as they were beginning conjugation; the component individuals of these three split pairs were called 1, *a* and *b*; 2, *a* and *b*; 3, *a* and *b*. Three other pairs, designated 6, 7 and 8, were allowed to finish conjugation, then their members (*a* and *b*) were isolated and cultivated on slides side by side with the others. Thus we have six lines that have just conjugated; six others that were attempting to conjugate, but were prevented.

The experiment may be divided into three stages. The first stage is from March 6 until April 11 (37 days). (Those that had conjugated did not divide until March 6, so that the fissions of those that had not conjugated are counted, for comparison only, from that day also.)

All the six lines of those not allowed to conjugate lived and multiplied vigorously throughout this period of five weeks and two days. Of the six lines derived from those that had conjugated, on the other hand, four died out completely within two weeks, while the others multiplied more slowly than did those that had not conjugated. The detailed records for the 12 lines are given in table 13. Here the classification by weeks begins March 8, so as to omit in the weekly record the irregularities due to the first two or three days after conjugation; this gives us just five weeks.

As table 13 shows, even the two lines of conjugants that lived multiplied less rapidly than did those that had not conjugated, the weekly average for all the former being 8.8, while for the latter it is 10.2. The next stage of the experiment was of a character to determine whether this difference in rate of fission continues beyond five weeks, as well as to decide whether it might be due to accidental causes, or was a result of inherent differences. Of the non-conjugants, the pairs 1 and 3 were continued till April 12 (31 days additional), while the conjugants were represented by 7 *a* only. Several lines of each set were kept in progress. The results are given in table 14 for two periods of

two weeks each, also for the total, thirty-one days; and for the entire sixty-eight days from the beginning.

As table 14 shows, the progeny of the conjugant line 7 *a* still reproduced somewhat more slowly than did the non-conjugant lines, during these last four weeks of the nine weeks during which the experiment had lasted. The average in the conjugants is less in each of the partial periods, as well as in the period as a whole. The average rate of fission is somewhat less in all lines than during the first five weeks; this is a common result of continued cultivation on slides.

In the third portion of the experiment two of the lines of non-conjugants (1 *a* and 1 *b*) and one of the lines of conjugants (7 *a*)

TABLE 13

Experiment 6. Paramecium aurelia. Comparative number of fissions in six lines derived from conjugants and in six derived from non-conjugants (split pairs), from a watch glass culture of the race k; for five weeks and two days. The fissions are given by days for the first nine days; for the rest only by the week. The numbers give the number of divisions that occurred in the time specified. The first week is counted from March 8 to March 14. (d = died out.)

		DAILY RECORD; DAYS OF MARCH											RECORD BY WEEKS					TOTAL 37 DAYS
		6	7	8	9	10	11	12	13	14	1st	2d	3d	4th	5th			
A. Those that have conjugated:																		
6 a.....			1	2	1	1	0	0	0	d	d							
b.....			1	1	2	0	1	0	1	d	d							
7 a.....			1	1	2	2	2	1	1	0	9	7	7	9	9	42		
b.....	d										d							
8 a.....			1	2	0	0	d				d							
b.....			1	1	2	2	1	2	1	1	10	7	10	10	10	48		
Mean per week, 8.80; mean for 37 days, 45.00																		
B. Those that did not conjugate																		
1 a.....		1	2	2	1	2	1	2	2	0	10	11	10	13	11	58		
b.....		1	1	2	1	2	2	1	2	1	11	6	11	12	10	52		
2 a.....		1	2	1	2	2	1	3	1	1	11	10	11	13	11	59		
b.....		1	1	1	2	2	1	1	1	1	9	9	9	13	9	50		
3 a.....		1	2	1	2	1	2	2	1	1	10	11	10	11	9	54		
b.....		1	1	1	2	1	2	2	1	0	9	9	9	8	10	47		
Mean per week, 10.20; mean for 37 days, 53.33.																		

were cultivated six weeks longer, making a total of sixteen weeks or 112 days. The comparative rates of reproduction for this period, as well as for the entire time, are shown in table 15. One point in this table requires explanation. During this period of forty-four days there were left of each set for various purposes separate lines of propagation which lasted less than the total period; for example, one line was continued seven days, another twelve, et cetera. These diverse periods have been summed for

TABLE 14

Experiment 6. Comparative rates of fission for certain lines that had conjugated March 4, and for others that were prevented from conjugating at that time. The number of fissions is given by periods of two weeks, for weeks 8 to 9; also for the last 31 days of a period of 68 days; and for the entire period.

	WEEKS 6 AND 7	WEEKS 8 AND 9	LAST 31 DAYS	TOTAL 68 DAYS
A. Conjugant				
7 a (line 1).....	13	14	30	72
(line 2).....	13	15	30	
(line 3).....	15	11		
Mean for 7a.....	13.7	13.3	30	72
B. Non-conjugant				
1 a (1).....	15	16	35	88
(2).....	15	17	36	94
(3).....	13	14	31	
(4).....	15			
Mean.....	14.5	15.7	34	91
1 b (1).....	18	15	37	88
(2).....	15	15	35	
(3).....	20			
(4).....	18			
Mean.....	17.75	15	36	88
3 a.....	17	13	36	90
3 b (1).....	12	15	31	78
(2).....	13	16	32	
(3).....		14		
Mean.....	12.5	15	31.5	78
Mean of means for all non-conjugants.....	15.94	14.68	34.38	86.75

each set, and are presented, with the total number of fissions during the periods, in the entry numbered 2.

Examination of table 15 shows that the line 7 *a.* derived from conjugant, no longer differs in any very marked or constant way in its rate of fission from the two derived from the non-conjugants; it is certainly not slower in its rate than the others. So far as the experiment goes, it indicates that after about two months the rate of fission of the conjugants, which had been made slower by conjugation, has regained about the usual rate. Owing to the small numbers of diverse lines involved, such a conclusion is of course not very secure.

TABLE 15

Experiment 6. Paramecium aurelia. Relative numbers of fissions in certain periods, and rates of fission, in certain conjugant and non-conjugant lines, for the last 44 days of an experimental slide culture that lasted 112 days from the time of conjugation; also totals for the entire 112 days of the experiment.

	CONJUGANT LINE 7 <i>a</i>			NON-CONJUGANT LINE 1 <i>a</i>			NON-CONJUGANT LINE 1 <i>b</i>		
	Days	Fissions	Daily rate	Days	Fissions	Daily rate	Days	Fissions	Daily rate
A. Changed every 48 hours during last 44 days									
1. Single consecu- tive line.....	44	48	1.091	44	38	.864	44	45	1.023
2. Sum of diverse periods for parts of line...	157	155	.987	141	123	.872	135	139	1.030
3. Single consecu- tive line from beginning.....	112	120	1.071	112	133	1.188	112	133	1.188
B. Changed every 24 hours during last 44 days:									
1. Single consecu- tive line.....	44	62	1.409	32	40	1.250	44	60	1.364
2. Sum of diverse periods, for parts of line...	110	176	1.557	88	115	1.307	133	176	1.323
3. Consecutive line from beginning	112	134	1.196	100	128	1.280	112	148	1.321

The experiment as a whole shows the fact that after conjugation the organisms are in a condition such that many may die, while those that have not conjugated live; and the further fact that the rate of reproduction is made slower by conjugation,* remaining in this condition for about two months. This is true even when all the lines concerned belong to the same race (derived originally from the same single individual).

Experiment 7: March 29 to April 10, 1910: Paramecium aurelia

On March 29 there was a conjugation in the progeny of a single ex-conjugant of January 29; the relation of these to the remainder of the experiments will be seen from the diagram, figure 1. These animals belong to the branch *B* of the diagram. They have conjugated once, and probably twice, since those of the branch *A*, which are known not to have conjugated since some period before March 4, and to have been ready for conjugation March 4. A comparison as to rate of fission was made between these non-conjugants of branch *A* and the conjugants of March 29, branch *B*, lasting for nine days (April 1 to April 10).

Of the conjugants of branch *B*, 19 lines were in progress; their average rate of fission per line for the nine days was 1.409 per day. Of the non-conjugants (branch *A*), 21 lines were in progress; their mean rate for the nine days was 1.455 per day.

Thus the non-conjugants of branch *A* give no indication as yet of injury through having omitted conjugation. The difference in rate between conjugants and non-conjugants was slight, but in favor of the non-conjugants.

Experiment 8: April 9 to April 30, 1910: Paramecium aurelia

On April 9 there was conjugation among the progeny of one of the ex-conjugants of Experiment 7 (conjugation March 31) shown in branch *B* of figure 1. There have now been seven generations of inbreeding in this branch; and it has conjugated twice (probably three times) since those of branch *A* have conjugated at all. What difference will this make between the rate of fission in the members of the two branches?

Eight lines descended from these conjugants of April 9 (branch *B*) were kept under observation till April 30. As the first fission did not occur till April 12, this gives nineteen days during which the rate of fission was determined for these. Of the non-conjugants of March 4 (branch *A*, fig. 1), fifteen lines were in progress at this time.

The average number of fissions for the nineteen days was, in the conjugants of branch *B*, 21.375, while the average rate of fission was 1.125 per day. In the non-conjugants of branch *A*, the average number of fissions for nineteen days was 21.533, the daily rate 1.133.

There was thus no appreciable difference in rate of fission. The branch *A* shows no sign of injury as a result of having omitted several conjugations which the branch *B* has undergone.

Experiment 9: April 29 to June 7, 1910: Paramecium aurelia

On April 29 there was another conjugation in branch *B* (9, fig. 1) in the same direct line as the conjugations of Experiments 7 and 8. That is, the conjugants of our present experiment are all descended from a single ex-conjugant of Experiment 8, these from a single ex-conjugant of Experiment 7, and so on. Thus there have now been in this branch *B* three conjugations (probably four), since there has been a conjugation in branch *A*. The members of branch *A* have been cultivated on slides since March 4, while those of branch *B* have been cultivated part of the time on slides, part of the time in watch glasses.

Precise comparison of branch *A* (fig. 1) with the progeny of one of the ex-conjugants of April 29 in branch *B* was not made till about two weeks after the conjugation of the latter. During this time observation with the eye seemed to indicate that the members of *B* were a little larger than those of *A*. In view of this apparent differentiation between the two, experiments were set on foot for comparing the fission rate and the dimensions.

Fission rate. Four separate series or lines of propagation were carried on from May 22 to June 7, both for the conjugants (*B*) and the non-conjugants (*A*).

We may divide this time into two periods of eight days each. The results for the two sets are given in table 16, the four parallel lines of each set being numbered (1) to (4).

The table shows that for the total period, each of the four lines of *A* multiplied more rapidly than any of the four lines of *B*. For any of the eight-day periods, the lowest record for *A* is at least equal to the highest for *B*, save in one single case. The lines of *A* average for the entire period 20.5 per cent more fissions than those of *B*.

When we recall that *B* has conjugated recently, and three times since *A* has conjugated at all, we see that the dropping out of the conjugations has not unfavorably affected the rate of reproduction in *A*.

Dimensions. To the eye it appeared that *B* was a little larger, under the same conditions, than *A*. As these belong to the same pure strain, this is a matter of interest, as it would show that hereditary differences in size may arise within the pure strain possibly as a result of conjugation. A careful comparison of the dimensions was therefore made. Keeping all under the same cultural conditions, I first measured a number of individuals of each set at the same age, choosing the age of thirty minutes after fission. Three other measurements were taken; the results of all are given in table 17.

The fact that, as table 17 shows, *B* was larger at each of the four measurements, seems to indicate that there has indeed arisen a slight hereditary differentiation in size within the pure

TABLE 16

Experiment 9. Paramecium aurelia. Comparative number of fissions May 22 to June 7, for two sets, one of which (B) has conjugated three times in series since the other (A).

		FIRST 8 DAYS	SECOND 8 DAYS	TOTAL			
		FIRST 8 DAYS	SECOND 8 DAYS	TOTAL			
B. Line (1)	13	8	21	A. Line (1)	15	11	26
(2)	14	5	19	(2)	15	9	24
(3)	14	7	21	(3)	14	13	27
(4)	14	8	22	(4)	11	12	23
Mean	13.75	7.0	20.75	Mean	13.75	11.25	25.00

TABLE 17

Experiment 9. Paramecium aurelia. Comparative lengths in microns of A and B. These belong to the same pure strain, but A has been cultivated for a long time without conjugation, while B has conjugated at least three times in succession since A.

	No.	Mean length	No.	Mean length
May 19: age, 30 minutes.....	10	116.600 \pm 0.551	11	125.454 \pm 1.063
May 22: adults, ill fed.....	46	99.000 \pm 1.195	50	123.680 \pm 0.913
May 28: adults, well fed.....	64	135.469 \pm 1.042	70	141.429 \pm 0.785
June 1: adults.....	65	131.877 \pm 0.616	41	132.237 \pm 0.747

line *k*. However, the fact that the difference was so extremely small at the last measurement taken admonishes us not to lay too much stress upon this case; the matter must be tested further.

Determination of conjugation. With *A* and *B* (fig. 1) a study was made as to the relative influence of external and internal conditions in inducing conjugation. In *B*, as we have seen, there had been at least four successive conjugations since there has been one in *A*. Will *A* be therefore readier to conjugate than *B*? If conjugation depends mainly upon an internal condition of need, then certainly we should expect this.

To test this, watch glasses of *A* and *B* were set side by side May 13, two weeks after the last conjugation of *B*, and the conditions for inducing conjugation supplied, so far as possible. On June 3 conjugation occurred in both *A* and *B*.

Thus under the proper conditions both sets conjugate at the same time, in spite of the fact that one has conjugated at least four times since the other. The experiment indicates that the recent external conditions are of more importance in determining conjugation than a progressive internal need arising through the fact that conjugation has not lately occurred.

Experiment 10: May 10 to May 20, 1910: Paramecium aurelia

On May 10 there was a conjugation in branch *C* (fig. 1, page 307), making the third in series in this division since any conjugation occurred in *A*. Four pairs were isolated from this new conjugation in branch *C*, and experiments were set on foot for

comparing these as to vitality, reproductive power, et cetera, with *A* (which had not conjugated for some months), also with those of branch *B* (which had conjugated two weeks before).

The four ex-conjugants of two pairs of *C* were placed on slides and treated like the non-conjugants of branch *A*. Two of these ex-conjugants divided once, two did not divide at all, and all died after three to ten days. Meanwhile, the members of branch *A* multiplied actively, at about the rate of once per day.

Two other pairs from branch *C* were allowed to multiply in a watch glass. This they did very slowly, so that on May 13 but 10 individuals had been produced from the four. These were then carefully brought into identical conditions with an equal number of specimens of *A*, and of *B*, the three being placed side by side in watch glasses.

On May 16, all the specimens of *C* were dead, while the members of *A* and *B* were flourishing.

Thus in this case, the recent conjugants (*C*) multiplied very slowly or not at all, and soon died; while others that had not conjugated so recently nor so often (*A* and *B*) flourished.

Experiment 11: June 3 to July 28, 1910: Paramecium aurelia

Comparison of conjugants and non-conjugants of the branch *A* (fig. 1, page 307).

On June 3 there was conjugation in a watch glass (taken from the slides May 15) of members of the branch *A* (fig. 1), derived from a split pair of March 4. Other divisions of this same stock (branches *B* and *C*, fig. 1) had conjugated four times in succession since any conjugation in *A*. Thus we have in *A* a set that has gone long past the normal conjugation period. Part of it now (June 3) conjugates, while the remainder (part in the watch glass, part on slides) does not. Thus we have an opportunity to test the effects of conjugation on the vitality and reproductive power of a stock that has long gone without it. Experiments for this purpose were conducted on slide cultures, and also in watch glass cultures.

To make clear the conditions in these experiments (which when described in words alone are a little confusing), I give in

figure 2, a diagram, which shows the relations of the various parts of the experiment. If the reader will make frequent references to this, he will have no difficulty in following the account of the experiments, and appreciating their bearings.

We have, derived from the branch *A* of figure 1, at first two divisions, designated *D* and *E* in figure 2; *D* has been cultivated continuously on slides, since March 4, while *E* was transferred on May 15 from the slides to a watch glass.

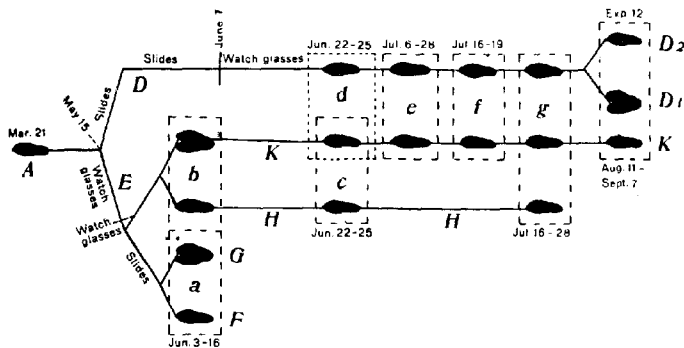


Fig. 2. Diagram showing the history and the nature of the comparisons made in the various divisions of Experiment 11, and in Experiment 12. Each rectangle represents one of the parts of Experiment 11, save the one to the right, which represents Experiment 12. The lower case letters *a* to *g*, within the rectangles, show the letters under which the various parts of Experiment 11 are described in the text; thus the one marked *e* shows Experiment 11 *e*. The capital letters *A* to *K* show the diverse branches of the race *k*, described in the text. The duration of the experiments is given by the dates below or above each rectangle. The legends at the branches *D*, *E*, etc., show the manner of cultivation; the branches *D* and *E* being separated on May 15, the former placed on slides, the latter in watch-glasses, etc.

Now, on June 3, this branch *E* is divided into four parts, which we may call *F*, *G*, *H* and *K*. The first two are kept on slides; the second two in watch glasses. The lots *F* and *H* are non-conjugants, while *G* and *K* are conjugating pairs kept under the same conditions. Comparison of *F* and *G* we may call Experiment 11 *a*; comparison of *H* and *K*, Experiment 11 *b*.

Experiment 11 a: cultivation on slides. From the watch glass (fig. 2, *E*), thirty-two ex-conjugants, from 16 pairs (*G*, fig. 2) and thirty-two non-conjugants (*F*, fig. 2) were isolated. These 64 lines were cultivated side by side, on slides, under identical conditions.

In both sets the mortality was high (as I have invariably found to be the case in attempting to cultivate *Paramecium aurelia* in hot summer weather). By June 13, 25 of the 32 lines of ex-conjugants (*G*) had died. By June 16 all the ex-conjugant lines were dead, while nine of the non-conjugant lines (*F*) were still alive.

In the ex-conjugants the average number of fissions, for those that lived to June 13, was 5.445, while in the non-conjugants, for the same period, it was 7.857. The average rate of fission for all the ex-conjugants (reckoning the rate for each one as long as it lived), was 0.5485 per day, while for the non-conjugants, reckoned the same way, the average rate was 0.7365 per day; so that the rate for the non-conjugants was 34.27 per cent greater than for the ex-conjugants.

Experiment 11 b: cultivation in watch glasses. Parallel to the slide cultures of 11 *a*, two watch glass cultures, one of conjugants (fig. 2, *K*), one of non-conjugants (*H*), were propagated. One of these contained at the beginning 30 ex-conjugants, the other 30 non-conjugants. On each of the following days the animals were removed one by one to a new watch glass, counted, and the number reduced so as to be the same for each. The ratio of the number present on each day to the number present the day before was thus obtained, this may be called the multiplication ratio. It was as follows for eight successive days, beginning June 5:

DAY	1	2	3	4	5	6	7	8	MEAN
Progeny of conjugants (<i>K</i>).....	1.67	1.84	2.17	1.10	1.11	1.26	1.47	1.51	1.52
Progeny of non-conjugants (<i>H</i>).....	2.80	2.08	1.92	1.52	1.80	1.80	1.74	1.56	1.90

Thus the rate for the progeny of the non-conjugants was greater every day except one; and the mean rate for the non-conjugants was almost exactly 20 per cent greater than for the progeny of the conjugants.

It is clear therefore that the progeny of the non-conjugants have a great advantage, both as to rate of reproduction and as to mortality.

Experiment 11 c. The two sets in the watch glasses (fig. 2, *H* and *K*) were allowed to multiply till June 22, then 10 individuals were removed from each, isolated on slides, and their rates of fission followed individually and compared. All multiplied vigorously, in two days 8 of the conjugant progeny had divided five times; 1 three times, 1 six times. Of the non-conjugant progeny, 5 had divided six times, 2 five times, 2 three times, while 1 died. Thus the two are now nearly equally vigorous, the rate of fission being still a trifle higher for the non-conjugants.

Experiment 11 d. The non-conjugants dealt with thus far (Experiments 11, *a* to *c*) had come originally from the same watch glass as did the conjugants (that is, from branch *E*, fig. 2). But there was under propagation at the same time, another set of non-conjugants (branch *D*, fig. 2), cultivated on slides since March 4, while those just described (*F* to *K*, fig. 2) had been cultivated in watch glasses (hence with more fluid) since May 15. On June 7 a watch glass culture of this slide series (*D*, fig. 2) was made, and left uniform with a watch glass culture (branch *K*, fig. 2) derived from the conjugants of June 3. On June 22, 10 individuals were taken from each of these two watch glasses (*D* and *K*, fig. 2), and cultivated on slides, in order to compare their fission rates and general vigor. The results were strikingly different from those thus far obtained; on account of their great interest I give them in detail.

Table 18 shows that, contrary to all our previous results, the progeny of the conjugants *K* are much more vigorous than those of the non-conjugants *D*.

Now, in the experiment which just preceded this (11 *c*), we saw that in a test made on the same date as the present one, and with conditions identical, the non-conjugants (*H*) with the same history

TABLE 18

Experiment 11 d. Paramecium aurelia. Number of fissions for three successive days, in ten non-conjugants from the slide series D (of fig. 2), and in ten progeny of the conjugants (K, fig. 2), that had lived in watch-glasses since May 15 (d = dead).

LINE	NON-CONJUGANTS (D)				CONJUGANTS (K)			
	June			Total	June			Total
	23	24	25		23	24	25	
1.....	1	1	d	(2)	3	2	2	7
2.....	1	0	d	(1)	d			(0)
3.....	1	d		(1)	3	2	1	6
4.....	d			(0)	3	2	1	6
5.....	0	d		(0)	3	2	1	6
6.....	2	0	0	2	3	3	1	7
7.....	2	0	0	2	3	3	1	7
8.....	d			(0)	3	2	2	7
9.....	0	d		(0)	3	2	2	7
10.....	2	1	0	3	2	3	3	8

as the conjugants (K) (cultivation in watch glasses since May 15) were not less vigorous than the progeny of the conjugants, but at least equally vigorous with them. The only difference between the non-conjugants of this present experiment (D) and those of the previous one (H) is that those in the present experiment were cultivated about a month longer on slides (May 15 to June 7). Apparently this is the cause of the weakness of these animals.

But it is clear that the progeny of the non-conjugants of this slide series D are now in a weakened, depressed condition, while both conjugants and non-conjugants from watch glass cultures are vigorous. This gives us an opportunity to determine the effects of conjugation in such a depressed culture. Before undertaking this, two additional tests were made to see if the depressed condition of the progeny from the slide series D was beyond doubt.

Experiment 11 c. The first of these consisted again of ten lines of the non-conjugant slide series D, ten from the conjugants (watch glass series, K). The experiment continued from July 6 to July 28.

In this experiment, as soon as any line of either set died out, it was replaced from some other line of the same set. The number

of such necessary replacements will give a comparative measure of the mortality in the two sets. In the non-conjugants *D* (slide series) there were necessary 34 such replacements; in the conjugant progeny *K* (watch glass series), there were 22. Of the conjugant line (*K*) six lived to the end (21 days), the average number of fissions for these being 21. Of the non-conjugant lines (*D*), four were alive at the end, their average number of fissions being 16.25. On the sixteenth of July ten lines were alive in each set; the average number of fissions at that time was for the conjugants (*K*) 15.6; for the non-conjugants (*D*), 13.00.

Experiment 11 f. On July 16, an additional comparison was made, taking fourteen each of the conjugants (*K*) and non-conjugants (*D*). In the nature of the results this experiment lasted but a short time. Of the non-conjugants *D* (slide series), only two divided, and all were dead by the third day. Among the conjugants (*K*), all divided; three died out during the three days; the remaining 10 averaged six fissions in three days. The non-conjugants (*D*) are clearly depressed and weak.

Experiment 11 g. A crucial question is whether the two sets of non-conjugants of unlike history—the slide series *D* and the watch glass series *H*—are still unlike in their vigor, as comparison with the conjugants (Experiments 11 *a-f*) indicates. Therefore, on July 16 comparative tests were made of these two, and also of the progeny of the conjugants of June 3 (*K*).

The three lines compared are those designated *D*, *K*, and *H*, figure 2. Of line *D* (non-conjugants cultivated on slides up to June 7) two watch glasses, containing five specimens each, were taken. Of the other non-conjugant line *H* (cultivated in watch glasses since May 15), the same number was propagated, in the same way. Of the ex-conjugants of June 3—the line *K*, with the same cultural history as the non-conjugants of line *H*—three watch glasses of five specimens each were propagated. In all three series the conditions were made exactly alike, all the animals being washed in the same water before they were introduced into the culture fluid. At intervals of some days the animals were removed one by one to a new watch glass, counted, and the number reduced, so as to be the same in all. The ratio of those

present to those that had been introduced was taken at each; this gives the ratio of multiplication for the two days. These ratios are given in table 19.

From the results given in table 19 the following are clear: (1) the non-conjugants, *D*, cultivated on slides till June 7, are still much depressed, and much less vigorous than either the non-conjugants (*H*) which came from the watch glass with the conjugants, or than the conjugants themselves (*K*); (2) the non-conjugants (*H*) which have the same cultural history as the conjugants (*K*) are still somewhat superior in vigor to the conjugants. In every one of the four periods their ratio of multiplication is greater.

The results of this experiment are then throughout consonant with those of Experiments 11 *a*, 11 *b* and 11 *c*. From all, the

TABLE 19

Experiment 11 g. Paramecium aurelia. Ratio of multiplication for a number of periods of time, in watch glass cultures of the three sets D, H and K, described in the text.

	JULY 16-18	JULY 18-20	JULY 20-24	JULY 24-28
(D) Non-conjugants, cultivated on slides till June 7.				
Culture 1.....	1.8	1.778	7.3	2.5
Culture 2.....	1.	1	<i>d</i>	<i>d</i>
Mean.....	1.4	1.389	3.65	1.25
(H) Non-conjugants from same watch-glass as the conjugants of K, set May 15.				
Culture 1.....	2.8	2.857	6.5	6.2
Culture 2.....	4.02	2.476	5.5	10.0
Mean.....	3.41	2.667	6.00	8.1
(K) Conjugants of June 3, from same watch-glass as the non-conjugants of H.				
Culture 1.....	3.00	1.533	3.9	6.6
Culture 2.....	2.00	2.110	6.2	3.7
Culture 3.....	3.00	1.867	5.9	6.4
Mean.....	2.667	1.833	5.333	5.567

conclusion is evident that the reason why the conjugants (*K*) are more vigorous than the non-conjugants (*D*) of the slide series, is because the latter were cultivated for a month longer on slides. For the non-conjugants (*H*), cultivated throughout in the same way as the conjugants, are still more vigorous than the latter.

To this the only objections that could be raised in favor of the view that conjugation has caused rejuvenescence would be as follows: (1) It might be held that it is not possible to be certain, in taking free specimens from a watch glass containing conjugants, that one is not taking specimens that have already conjugated. I believe that there is no ground for this objection in the present case, since the cultures in question were watched with the utmost care in order to detect conjugation at its beginning. Further, Experiments 11 *a* and 11 *b* show that there was an actual difference between conjugants and non-conjugants, of the same sort as that found in all our other experiments, the non-conjugants being the more vigorous. It is clear therefore that in this case we were actually dealing with non-conjugants. (2) It might be said that it would be difficult to be certain that in the period that had elapsed since June 3 there had not been conjugation in the vessels of 'non-conjugants.' It may be admitted that this difficulty exists, though in this case the organisms were inspected daily, and I believe that no conjugations occurred. But this objection is in fact negated by the experimental results themselves. The greater vigor of the non-conjugants seen in Experiment 11 *g*, of July 16, is of just the same character as that seen immediately after conjugation (Experiments 11 *a* and 11 *b*) and as that characteristic of the non-conjugants in all our other experiments, to which the present objection is not applicable.

But is the explanation given above—the fact that the non-conjugants of one set (*D*) were cultivated a month longer than the other (*H*) on slides—a credible one? I believe that everyone who has had extensive experience with experiments of this sort will agree with me that long continued cultivation on slides does produce a depressed condition. There are some stocks that will not stand it at all, though they live perfectly in mass culture. And in fact, my experimental records show that in this partic-

ular stock of *A* (fig. 1) those cultivated on slides became very unhealthy in June, so that all died out June 18; the branch was preserved only because some of them had thus been removed to watch glasses on June 7, forming our present stock *D* (fig. 2).

Thus all the evidence, from many distinct sources, points to the explanation we have set forth above, that the non-conjugants of the slide series *D* are less vigorous than either the non-conjugants (*II*) or the conjugants (*K*) of the watch glass series, because they were cultivated longer on slides.

Experiment 12: Conjugation in a depressed stock: August 10 to September 7: Paramecium aurelia

Whatever the cause, we now have on hand, after the Experiments 11 *a* to 11 *g*, a much depressed stock, which has omitted at least four normal conjugations. Now, it might be maintained that our uniformly negative results thus far as to rejuvenescence by conjugation are due to the fact that we were not dealing with depressed stocks. It is of interest, therefore, to determine the effects of conjugation within a stock thus known to be depressed. There is, however, great difficulty in carrying out such an experiment, for such depressed stocks cannot easily be induced to conjugate. The main condition for conjugation appears to be, that there shall be a period of rapid multiplication, followed by a decline in the conditions inducing it. But in such depressed stocks it is almost impossible to induce rapid multiplication. After many efforts, I finally succeeded on August 10 in getting a scanty conjugation in a watch glass culture of this depressed set of the slide-series of *D* (fig. 2) which has descended without previous conjugation from the split pair of March 4. I was able to obtain for study but three pairs, the ex-conjugants of course forming when isolated six lines of propagation. I call these sets *D* 1.

From the same watch glass culture I isolated at the same time ten of the individuals that were not conjugating, and from these, ten lines of propagation were derived, which were kept under the same conditions as those from the conjugants. These may be called *D* 2.

For further comparison, I placed beside these, ten lines derived from a culture of this same series that had conjugated June 3, and had lived in small mass cultures since. These belong to the line *K*, figure 2.

As this experiment gives the only results obtained that could be interpreted as showing a favorable influence of conjugation on survival and reproduction, it appears best to give in some detail the records for the lines of propagation. This is done in table 32, in the Appendix.

To understand table 32, the following must be considered: Each line of any of the three sets was started with a single individual August 11. The animals were changed to new fluid every day or every other day (all being alike), and all those that had been produced were retained, save on certain days, when the number was reduced, by removal of certain of the animals. To give the essential facts in the history of the cultures, it is therefore necessary only to give the number of individuals on these dates, before and after reduction. This is what is done in table 32. Thus, in line 1 of set *K*, the single individual of August 10 had on August 14 produced 6, of which 4 were removed, leaving 2. On August 16, these 4 had produced 8, of which all but 2 were removed, et cetera.

The last column of table 32 gives the total number of fissions undergone by the line in question, up to September 7, or to its death. As the lines of set *K* were all obviously vigorous, only five were kept under observation till September 7, when the last line of set *D* 2 died out.

To grasp the results, it will be best to examine first the facts for set *K*, which had lived in mass cultures since May 15, and had conjugated June 3. In this case, as will be observed, all the ten lines flourished well. Number 4 was lost by accident August 28, and numbers 3, 5, 7 and 9 were discontinued September 3, because the results were clear.

Now, compare with these the results given for the conjugants and non-conjugants of August 10 (the depressed race) in sets *D* 1 and *D* 2. It is clear from the data of set *D* 1 that the conjugation of August 10 has by no means restored this depressed series to the

level shown by the other set *K* of table 32. In spite of the utmost care, and the division of the lines as soon as possible, so as to have more than one from each of the ex-conjugants, the progeny of three of them had died out within eight days, and a fourth died out later. Only two of them survived till September 7, when this experiment was discontinued; and the nine lines derived from these two were then multiplying much less rapidly than those derived from set *K* (table 32). It should be stated further that two tests made respectively three and four months later (one December 1, 1910, the other January 7, 1911), showed that the members of set *K* (conjugants of June 3) were still far more vigorous than conjugants of August 10 (set *D 1*). In the test comparison of December 1, all the twelve lines of set *D 1* (conjugants of August 10) died out after a week of cultivation on slides, while those of set *K* flourished.

When however we compare the records for the conjugants of August 12 (set *D 1*) with those for the non-conjugants of the same culture (set *D 2*) in table 32, we find that the conjugants have a decided advantage. The non-conjugants ceased multiplication almost entirely, after the first week, and gradually died out, the last one dying on September 7. At this time the descendants of two of the ex-conjugants were multiplying well, so that an indefinitely large number of progeny were later produced from them.

Thus in this case two of the six conjugants were more vigorous than any of the non-conjugants of the same stock and cultural history. Most of the conjugants died out, but in the natural course of events the entire set would have been replaced by the progeny of the few more vigorous lines.

This is the only case, out of a very large number of experiments, that gives any indication of a beneficial effect of conjugation on vigor and survival. Just what has happened here? First, attention should be called to the fact, already set forth, that conjugation in this depressed stock was very scanty; in connection with the further fact that the condition for producing conjugation is a period of rapid multiplication, followed by a check. Now, from this and from the data of tables 18 and 19, it is evident that there

were few in this depressed race that could be induced to multiply sufficiently to furnish the conditions required for conjugation. Those that did conjugate evidently represent then *those members of the stock that are most vigorous and active in multiplication*. Their later vigor and survival, as compared with the non-conjugants, may therefore have been due to this, and not to the conjugation; in other words, conjugation may have been the effect, not the cause, of their greater vigor. If the same individuals that conjugated could have been cultivated without conjugation, it is probable that they would have multiplied equally well or better.

However this may be, it is clear that conjugation did not cause rejuvenescence in any simple direct way, since the majority of the conjugants died out, and those that survived were weak. But in one respect this experiment gives the same results as all others. Conjugation resulted in an increase of variability, as regards vigor and rate of reproduction. Among the extreme variates were some whose vigor was sufficient to keep them alive, while among the more uniform non-conjugants all died. The advantage of the conjugants, so far as it did not exist before conjugation, is then in this case due to the effect of conjugation in increasing variation.

Experiment 13: Production of inherited differentiation by conjugation: December 6, 1910, to May 15, 1911:

Paramecium aurelia

A very extensive and long-continued series of experiments was carried on in the winter and spring of 1910-1911, with the same pure strain *k*, of *Paramecium aurelia*, that was used in the experiments just described (Experiments 6 to 12). The main purposes of this new set were, to determine whether as a result of conjugation differentiations may arise within a pure strain, and to bring out the rules of inheritance within the pure strain. Most unfortunately, in the later and most critical part of the experiment the conditions became such that multiplication almost ceased, and this made futile a large part of the work, particularly that designed to discover the rules of inheritance. Whether this

cessation of reproduction was due to something in the cultural conditions, or to weakening of the stock as a result of long continued culture on slides is perhaps not absolutely clear, though the evidence is strong that the latter alternative is the correct one. But in spite of this, the experiment gave definite results on some important questions. I shall give the experimental data only so far as they throw light on definite problems.

From many other experiments the general impression had been obtained that conjugation produces inherited differentiation even within the pure strain. By 'pure strain' is meant here simply a series of animals all derived from one single individual. Experiments set forth in previous papers indicate that no inherited differentiation within such a pure strain arises, as a rule, during multiplication by fission; and this agrees essentially with most other work on inheritance in vegetative reproduction. The evidence, so far as *Paramecium* goes, was based mainly on studies of the inheritance of size. If, as these indicate, heritable differentiations do not arise in fission, then the question comes up as to how the existing differentiations into diverse races do arise. The indications just mentioned, that conjugation produces such differentiation, then of course call for investigation; this was attempted in the present series of experiments.

If inherited differentiation does result from conjugation, this might be held to be due to Mendelian inheritance, or something similar. If the individual with which the pure strain began was a heterozygote, and its progeny through fission were identical heterozygotes, then of course when these interconjugated, new combinations of various sorts might be produced, exactly as differentiations may arise by self-fertilization of heterozygotes in plants.

Eight self-fertilizations. To avoid, so far as possible, the heterozygotic condition, I used the race *k*, of *Paramecium aurelia*, already described in connection with Experiments 5. to 11 (see diagram of its history, fig. 1, page 000). At the time when the present series of experiments (13 and 14) begins, self-fertilization had occurred in this race eight times in series. That is, the progenitor of the race was a single individual; its progeny con-

conjugated among themselves; from these conjugants a single ex-conjugant was taken and allowed to multiply till there was conjugation among these. A single member of a pair was again allowed to propagate till there was conjugation; and thus the process was repeated eight times, all the members of each of the eight non-sexual series being the progeny of a single ex-conjugant of the previous series. The known history of this race is illustrated in the diagram of figure 1. This diagram shows also the relation of the organisms employed in the present experiment to those used in previous experiment. They belong to branch *B* of figure 1, and are derived from a single ex-conjugant of the conjugation of April 29; they are thus the same stock as the conjugants employed in Experiment 9.

Self-fertilization for eight generations in succession, of course goes far in getting rid of heterozygotism in most characters. East and Hayes ('12) have given the general formula for determining what proportion of the organisms would be homozygotic with respect to any given number of characters after a given number of self-fertilizations; this being based on the formula originally given by Mendel ('66). In a recent note, written before the paper of East and Hayes had appeared, I went into some details on the matter (Jennings '12). If we call x the proportion of the organisms that will be homozygotic, letting n be the number of successive self-fertilizations and m the number of pairs of characters, then the formula for use is .

$$x = \left(\frac{2^n - 1}{2^n} \right)^m$$

From this formula we find that after eight successive self-fertilizations the proportion of the organisms that would be homozygotic for any one, two, or more characters, up to ten, is as follows:

NUMBER OF CHARACTERS	PROPORTION HOMOZYGOTIC	NUMBER OF CHARACTERS	PROPORTION HOMOZYGOTIC
1.....	0.99609	6.....	0.97679
2.....	0.99220	7.....	0.97297
3.....	0.98833	8.....	0.96917
4.....	0.98447	9.....	0.96539
5.....	0.98062	10.....	0.96162

Thus, after eight self-fertilizations, more than 96 per cent of the organisms would be homozygotic with respect to all ten characters.

Of course we do not know on how many independently heritable characters depends the rate of fission (which was the characteristic chiefly examined). If it depends on not more than 10 such characters, the chances are thus at least 26 to one that we are dealing with a pure homozygotic organism, when we select a single individual after the eighth successive self-fertilization of the line.

The above analysis is based on the view that there is no separation of the zygotic constituents in the reproduction by fission, this being indicated by the evidence thus far brought forward. If it were not true, then we would expect the organisms constituting a pure strain (descended by fission from a single individual) to become more and more diverse as fission was repeated, for as any individual became homozygotic with respect to any character it could produce forever after only progeny that were homozygotic in that respect. The result would be in the course of 20 or 30 generations to produce a set of individuals, each of which was homozygotic with respect to all the characters it bore, though the different ones would have diverse homozygotic characters. Selection among such individuals would then give rise readily to diverse races; this is opposed to the evidence hitherto obtained.

The eighth of the conjugations in succession took place April 29, 1910. A single ex-conjugant gave rise to a culture, which propagated without admixture, till this experiment was begun, December 6, 1910. On the evening of December 5 a watch glass of the animals was taken from the large culture; on the following morning those in this watch glass were conjugating, while those that remained in the large culture dish were not.

Experiment 13 a. Fifty-two pairs were taken from the watch glass, 100 non-conjugants from the culture dish; all these were isolated on slides, in the way already described. Thirteen of the pairs were later lost by an accident. This left 78 lines derived from animals that had conjugated, 100 from animals that had

not conjugated. Of the conjugants 20 died during the first week; of the non-conjugants 18, leaving 58 and 82 respectively in the two groups.

RATE OF FISSION. The ex-conjugants, as usual, began dividing the second day after conjugation. Beginning for both sets at this time, daily records were kept of the number of fissions in each line. Table 20 gives the fissions for the first week, in each of the two sets. As in all our other experiments, the rate of fission was somewhat greater in those that have not conjugated.

TABLE 20

Experiment 13 a. Paramecium aurelia. Comparative number of fissions in conjugants and non-conjugants of the same culture, for a period of one week, beginning two days after the separation of the pairs.

	NUMBER OF FISSIONS										TOTAL	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION	
	0	1	2	3	4	5	6	7	8	9					10
Conjugant lines	4		1	2	8	4	6	12	14	5	2	58	6.155 ± 0.220	2.484 ± 0.106	40.350 ± 2.910
Non-conjugant lines					2	5	16	33	15	8	3	82	7.098 ± 0.093	1.246 ± 0.066	17.550 ± 0.953

VARIATION. As in other cases, the variation in the rate of fission is much greater among the descendants of the conjugants than among those of the non-conjugants. The standard deviation is twice as great, and the coefficient of variation two-and-a-half times as great, in the descendants of the conjugants (table 20).

MORTALITY. Of the 78 conjugant lines, 20 died out during the first week, or 25.6 per cent. Of the 100 non-conjugant lines, 18 died out, a mortality of 18 per cent.

Experiment 13 b: inherited differentiations in the pure strain. At the end of this first week, those lines of each set that showed indications of differentiation in rate of fission were selected for farther propagation. That is, from both the conjugant set and the non-conjugant set the extreme lines were taken; also certain of the intermediate ones. Thus, from the conjugant lines there were selected the four that had not divided at all (table 20), the

one that had divided but twice; two whose record had stood at 3 fissions, six at 4, two at 5; then two at 8, two at 9, and the two at 10. In the non-conjugants, the two with a record of 4 were taken, three at 5, three at 6, four at 7, two at 9, and two at 10. In all, 21 of the conjugant lines and 16 of the non-conjugant lines were thus continued. Two of the former (15 and 16) were, however, derived originally from one ex-conjugant.

The purpose of continuing these 37 lines was to determine whether the varying rates of fission are inherited; this would show that inherited differentiation had arisen within the pure line, in this respect at least.

All but one of those conjugant lines which had not divided during the first week died out during the second week. Sixteen of the conjugant lines and fourteen of the non-conjugant ones were cultivated under identical conditions from December 6 to February 27, a period of ten weeks; a few of these lines died out, however, before the end of the period. It will be well to divide the period into five homogeneous divisions of about two weeks each, giving the fission rates for each line in each of these divisions. During the first four periods the organisms were changed daily; during the last one, every other day, a regimen under which they did not thrive. On this account, the first four periods are more characteristic and significant than the last. The fissions for these five periods are given in table 33 (Appendix). I have arranged them in the order of their relative rates of fission, as determined by comparing the total numbers of fissions in the first three periods (given in the last column).

Examination of table 33 shows clearly that in some cases at least the different rates of fission are inherited. Compare for example among the conjugants, line 4 and line 15 (or 16). In every one of the five periods, line 4 shows a higher rate of fission than does line 15. The same thing appears in other lines, of which details will be taken up later.

These constant differences appear in spite of the fact that all of the lines were treated in exactly the same way throughout the ten weeks' experiment. All were kept together, in the same moist chambers, and in the same culture fluid. In order that the drop belonging to one line should not have a continuously

different bacterial content from that of another, the animals of different lines were frequently interchanged; line 1 being transferred into a drop in which line 2 has been living, and vice versa. The drops were for the same purpose frequently intermixed.

If two such lines as No. 4 and No. 16 (of the conjugants) showed in the long run about the same rate of fission, but with accidental fluctuations from period to period, then of course in some periods No. 4 would show a greater number than No. 16, while sometimes the reverse would occur. When we find however that such a line as No. 4 has uniformly a greater number of fissions than another, and this continues for so long a time as ten weeks, with no external differences to cause these results, we must conclude that the lines themselves are differentiated.

We may make then as the test of inherited differentiation the condition that one line shall show in every one of the five periods of table 33 a distinctly higher fission rate than another. This is an extremely severe test, and one that is beyond question more than sufficient to show actual inherited differentiation. In the slow process of experimentation these repeated differences are most striking and surprising. Our first period covers eighteen days; during this time one finds that conjugant No. 1 divides more rapidly than No. 8 or No. 16. To test the matter, the three are kept under identical conditions for twelve days longer (second period). Again No. 1 shows the highest rate, No. 8 a lower one, No. 16 a still lower one. To make assurance doubly sure, we keep them fourteen days more (third period); again they show the same relative rates. We keep them a fourth period of twelve days; a fifth one of fourteen days; these confirm the differences shown in the first three periods. There can be no question but that the cause of the diversities is in the lines themselves; in other words there is differentiation inherited from generation to generation.

On this basis it is clear that among the conjugants, Nos. 1, 8 and 16 represent three lines with inherited differentiation in rate of fission. It is hardly doubtful but that other differentiated lines exist, accidental fluctuations bringing these equal to one

of the above three at one of the five periods. But we may hold rigidly to our test and still demonstrate the existence among the conjugants of the three diverse lines 1, 8 and 16. As will be observed, the fission rate is on the average more than twice as great in No. 1 as in No. 16.

Among the non-conjugants also there are inherited differentiations. In every period but one, non-conjugant line 1 has twice as high a fission rate as line 14. On the basis of our severest test, it is clear that lines 1, 12 and 14 are diverse in their inherited rate of fission.

It is clear therefore that heritable differentiations do arise within the pure line, so far as the rate of fission is concerned. How are these differentiations brought about?

At this point a weak spot in the plan of the present experiment appears. All our experiments show that conjugation increases the variability in the rate of fission; this is true both in wild cultures and in pure lines, and holds for the present experiment, as table 20 shows. It would appear probable therefore that some of these variations are inherited, and that this is precisely what the results given in table 33 demonstrate. But we find inherited differentiations also, as we have seen, among what we have called the non-conjugant lines of the present experiment. The weak point mentioned relates to the applicability of the term 'non-conjugant' to these lines. As already set forth, the last previous recorded conjugation took place for this line *k* on April 29. The present experiment began December 6. Now, it is almost certain that in the intervening time the animals had conjugated one or more times, since this race *k* conjugates once in one or two months, when conditions are favorable. Therefore, if conjugation produces differentiations, my 'non-conjugants' of the present experiment have had much opportunity to become differentiated in that manner; they are not properly 'non-conjugants' for present purposes. That they have become in some way differentiated is clearly shown by comparison of No. 1 with No. 14 in the non-conjugants of table 33.

This, of course, does not vitiate our main result, that inherited differentiation does arise within a pure line, and it leaves it

probable, or perhaps certain, that such differentiation arises in consequence of conjugation. But it leaves unsettled the question whether such inherited differentiations may not arise also in other ways.

To give clear results on this point, the experiment should have been performed as follows: A single individual of *k* should have been isolated, allowed to multiply by fission; watched continuously till the first conjugation occurred, then the experiment should have been performed with these conjugants and non-conjugants. If inherited differentiation appeared among the non-conjugants in such a case it could not be held to be due to conjugation.

These conditions are fulfilled in Experiment 15 on another race, to be described. They were likewise fulfilled in the latter part of the present experiment, and repeatedly in experiments on race *k* in 1912. But unfortunately race *k* has lost its power to flourish in slide cultures; in every case with the later experiments on this race all the lines have died out after a few weeks of culture. It would be of interest to carry out the experiments with race *k*, in view of its history of eight repeated self-fertilizations, and efforts will be made to find a successful method of slide culture for it. In the meantime the results of Experiment 15, with race *E*, give clear results on the main questions at issue.

The results of the present experiment therefore leave open the possibility that heritable differentiations may arise in other ways than by conjugation. Do they furnish positive evidence that heritable differentiation actually does arise as a result of conjugation? As we have seen, all our many experiments show that conjugation increases the variation in rate of fission between the lines. This is true (as already set forth) for the first week of the present experiment. Furthermore, if we compare the variability of the conjugant and non-conjugant lines of table 33, we find again that the conjugants are much more variable. We are of course not here dealing with random samples, but since both sets were selected to give as much variation as possible, a comparison of the variations may be of significance. The means, standard deviations and coefficients of variation for various periods are given

for the conjugant and non-conjugant lines, in table 21. The constants are given, not only for each of the five periods of table 33, but also for certain of these periods taken together; likewise for the first week (column 1).

Table 21 shows that: (1) in every case the mean rate is higher in the non-conjugants; (2) in every case the standard deviation (measure of the absolute amount of variation) is greater in the conjugants; (3) in every case the coefficient of variation (measure of the variation relative to the mean) is much greater in the conjugants.

Since these measures are based on the number of fissions for long periods under identical conditions, they can hardly be held to represent meaningless accidental fluctuations, but rather actual differentiations. They show further that these differentiations are much greater in those that conjugated during the last epidemic than in those that did not. This conjugation therefore caused inherited differentiations within the pure line. Whether the fewer inherited differentiations among those that did not conjugate during the last epidemic are due to previous conjugations we cannot tell in this case, but must refer the reader to the account of Experiment 15.

The inheritance of the rate of fission in these cases may be demonstrated, for those that prefer this method, by working out the coefficients of correlation. The numbers we are dealing with are of course small, but significant, owing to the great number of generations dealt with. We may take the fissions during the first two periods of table 33 and by determining their correlations with the fissions in the same lines for the second two periods, get a numerical expression of the inheritance. For the conjugant lines we find that the coefficient of correlation thus taken is 0.5031 ± 0.1346 . For the non-conjugant lines it is 0.5627 ± 0.1331 .

(A full treatment of the inheritance of the fission rate, by biometric methods, with adequate numbers, will be given in another connection; together with an analysis of the relation of this method of measuring inheritance to other ways of dealing with the matter.)

TABLE 21

Experiment 13 b. *Paramecium aurelia*. Comparative variability in fission rate for descendants of conjugants and of non-conjugants, for various periods. (The constants in the first column are based on the data of table 20; the remainder on the data of table 33, in the appendix).

	1	2	3	4	5	6	PERIODS 1 AND 2	PERIODS 3 AND 4	PERIODS 1 TO 5
	FIRST WEEK	FIRST PERIOD DECEMBER 8-26	SECOND PERIOD JANUARY 2-14	THIRD PERIOD JANUARY 15-29	FOURTH PERIOD JANUARY 30- FEBRUARY 12	FIFTH PERIOD FEBRUARY 13-27			
Mean-Conj.	6.153±0.220	12.647±0.608	9.250±0.263	11.188±0.629	9.789±0.608	6.167±0.762	22.750±0.913	21.214±1.126	33.875±1.243
Non-Conj.	7.098±0.093	15.786±0.520	10.143±0.235	12.857±0.653	11.417±0.436	9.364±0.550	25.786±0.561	25.083±0.982	38.786±1.149
Stand. Dev.—Conj.	2.484±0.156	5.551±0.642	1.561±0.186	3.733±0.445	3.707±0.473	3.912±0.539	5.414±0.646	6.247±0.796	7.373±0.879
Non-Conj.	1.246±0.066	2.883±0.307	1.301±0.166	3.622±0.462	2.253±0.310	2.706±0.389	3.668±0.468	5.041±0.694	6.372±0.812
Coef. Var.—Conj.	40.350±2.910	43.893±5.976	16.578±2.069	33.370±4.400	37.879±5.478	63.442±10.345	23.798±2.904	29.447±4.066	21.765±2.715
Non-Conj.	17.550±0.953	15.263±2.405	12.832±1.602	28.175±3.866	19.735±2.821	28.899±4.489	14.225±1.850	20.096±2.876	16.438±2.150

Experiment 13 c. In order to test more fully the inheritance of the differences in fission rate shown in table 33, certain lines were next selected for propagation on a more extensive scale. Beginning January 28, 1911, the attempt was made to propagate 16 parallel lines each of conjugant numbers 1, 2, 3, 11, 14 and 16 (of table 33), the purpose being (1) to determine whether the results with 16 lines of a given number confirm those obtained with but one line; (2) to discover whether there arise differentiations within any of the series derived from a single individual. This second point, as we have before seen, is fundamental for a full understanding of the results thus far reached.

These objects were not fully attained, owing to the cessation of active propagation on slides in the race *k*, but certain results of importance were reached.

CONJUGANT LINES. The sets derived from the different ex-conjugants of table 33 showed great differences in vitality as well as in rate of fission. Lines 1, 2, 3 and 11 began strongly, 16 parallel sets being derived from the original single set in one to three days. With conjugant lines 14 and 16, on the other hand, there was great difficulty in getting 16 sets established; multiplication was extremely slow, and many of the sets died out almost as soon as they were isolated. It was a week from the beginning of the experiment before 16 sets were in operation in conjugant lines 14 and 16.

The relative rates of fission that had characterized the various lines from the beginning continued to show themselves in the sets of 16 from each line. The slower lines showed much greater mortality than the faster ones. As fast as any set of a given line died out it was replaced from another set of that line. The number of deaths for each line was thus recorded. It will be instructive to give for each of these lines of ex-conjugants the number of fissions and the number of deaths, up to February 15. This is done in table 22.

The mortality in the slow lines increased from February 15 on, so that on February 16 there were but three sets left (out of 16) in line 14. By February 26 all the 16 sets of lines 14 and 16 were dead, so that these two lines became extinct. In the meantime,

TABLE 22

Experiment 13 c. Paramecium aurelia. Number of fissions, and number of deaths, in each of the sets of 16 parallel cultures belonging to six of the conjugant lines of table 33, between January 28 and February 15. For each line, the minimum and maximum number of fissions in the cultures that lived through the period are given. Thus, in line 1, one of the 16 sets gave 15 fissions, another 20.

	LINE					
	1	2	3	11	14	16
Fissions, January 28 to February 15.....	15-20	9-15	15-16	10-13	5-6	3-11
Number of deaths.....	1	4	0	8	19	20

the 16 sets of line 1 were flourishing under precisely the same treatment.

Later, lines 2 and 3 began also to die out. On March 7, line 2 was extinct, while of line 3 one set still existed. Line 1 continued to flourish; 16 sets still existed March 7. For other purposes 64 sets of line 1 were kept in propagation till April 17.

'NON-CONJUGANT' LINES. Later, the rapid line 1 and the slow line 14 of the 'non-conjugants' (of table 33) were compared similarly, beginning February 26. Sixteen sets of each were put in progress. Line 1 continued to multiply rapidly, line 14 slowly; by March 20 the maximum number of fissions in the former was 17, in the latter 6. At this date an attempt was made to increase the numbers to 64 parallel sets for each line. But it was found impossible to get 64 sets of line 14 into existence, owing to the great number of deaths. On March 31 the last set died out, and the non-conjugant line 14 became extinct. At this time there were 64 sets of line 1, which were continued till April 17, when the experiment was abandoned. During the last two weeks there were few fissions even in line 1.

Thus all of the lines having a slow rate of fission died out, even though the attempt was made to keep up 16 parallel sets; and this under conditions in which the lines with rapid fission continued to flourish. Although all were treated alike, only the two most rapid lines, No. 1 of the conjugants and No. 1 of the non-conjugants, continued to live till the close of the experiment, April 17.

This appears to indicate that the lines with slower fission are defective in some way. Of course it is possible, perhaps probable, that under more natural conditions they would have continued to exist, in spite of their slow multiplication. The extremely slow line 16 (conjugant) had lived from December 6 to February 26, a period of two months and twenty days, comprising forty successive generations. But slow multiplication and high mortality are decidedly correlated.

It had been planned to employ the 64 sets that were kept for a number of different lines in biometrical studies of the inheritance of the fission rate; and in an attempt to determine whether heritable differentiations in fission rate arise in the progeny of a single individual multiplying by fission. But the death of all the slow lines, and the extremely slow multiplication of the others for the last weeks of the experiment rendered the extensive data obtained valueless.

Summary of Experiment 13. We may summarize the results of this entire experiment as follows:

In a pure strain, all the individuals derived originally from a single one; and all derived from eight successive conjugations with self-fertilization of the strain:

1. Conjugation decreased the rate of fission.
2. Conjugation increased greatly the variability in rate of fission.
3. The differences in rate of fission were found to be inherited, so that in this respect heritable differentiations arise within the pure strain.
4. These heritable differentiations are due partly, if not entirely, to conjugation, since the latter increases greatly the variability. But whether such heritable differentiation may arise within the pure strain by other means is not determined in this experiment.
5. A low fission rate is correlated with a high mortality. Conjugation produces many lines with low fission rate; these lines die out in the course of time, if the conditions become severe, although the lines with rapid fission continue to live. But the slow lines may live for many generations (forty in this experiment).

Experiment 14: Paramecium aurelia

This was a direct continuation of the foregoing, dealing with the rapidly multiplying line 1 of the non-conjugants. On March 20, 1911, a considerable number of these were placed in a watch glass; on March 22, conjugating pairs were found among these. Of these 48 pairs were isolated, making after separation 96 lines, which were cultivated on slides as usual. At the same time there were in progress 64 lines of those that had not conjugated, and to these were now added 48 more. Thus we have now propagating, under identical conditions, 96 lines of ex-conjugants and 144 lines of non-conjugants, all derived from individual No. 1 of the non-conjugants of the previous experiment.

Conditions were unfavorable for multiplication, the temperature being low and the university buildings not heated. Of the 96 lines of conjugants, all but four died without dividing; that is, 95.8 per cent. Of the 48 non-conjugants set at the same time, 27, or 56.25 per cent died without dividing.

By March 31, nine days after conjugation, all but 4 of the 96 conjugant lines were dead, while 27 of the 48 non-conjugant lines were dead. Of the entire 144 non-conjugant lines, kept under the same conditions as the conjugant ones, 37 had died during the same period. Thus the proportion of deaths was, for the conjugants, 95.83 per cent; for the non-conjugants 25.69 per cent.

This experiment shows that under such unfavorable conditions the animals that have recently conjugated are much less resistant than those of the same descent that have not recently conjugated.

*Experiment 15: inherited differentiation produced by conjugation:
Paramecium caudatum*

In the summer of 1912, after several months spent in vain attempts to repeat with the race *k* the essential features of Experiment 13, under such conditions as would show beyond question whether all the inherited differentiations were due to conjugation or not, a successful experiment for this purpose was carried through with a race of *Paramecium caudatum* which I called *E*.

The race *E* was derived from a single individual taken July 31 from a wild culture of *Paramecium caudatum*. This individual and its progeny were allowed to multiply on slides till a large number were obtained. On August 19 many of these were transferred to a mass culture, and on August 22 watch glass cultures containing many individuals were removed from this mass culture. Early the following morning conjugation was beginning in these watch glasses. In the way set forth in our general account of methods (page 282), I picked out 67 pairs and 68 split pairs (pairs which had begun to unite, but which were separated before conjugation was consummated). The two members of each pair (and of each split pair) were designated *a* and *b*. The products of the first division of each of these were retained, becoming the progenitors of two lines which I called *x* and *y*. Thus from each pair (and each split pair), four lines were propagated, two from *a* and two from *b*. This of course gave 268 lines derived from conjugants and 272 derived from the non-conjugants of the split pairs.

During the heat of summer the cultivation of many lines of *Paramecium* is very difficult, owing to excessively rapid development of bacteria in the drop cultures. This has the effect of inducing a high mortality, and also of making it very difficult to keep the environmental conditions uniform throughout a large number of lines. This latter condition is essential in the present experiment, since if it is not fulfilled, differentiations in fission rate due to environmental conditions simulate those due to heritable or intrinsic differences in the diverse lines.

Owing to these difficulties the mortality among the conjugants was high, and the measures required for making the conditions uniform were so time-consuming that I was compelled to abandon a large number of the lines of propagation of the non-conjugants, so that I succeeded in keeping to the end of the experiment but 88 lines of conjugants, derived from 44 original ex-conjugants, and 174 lines of the non-conjugants, derived from 87 original members of split pairs. These however were sufficient for the solution of the problem that gave rise to the experiment. These 262 lines were propagated from August 24 to September 16

(non-conjugants) or September 18 (conjugants), a period of 24 (or 26) days.

Culture methods necessary to secure uniformity of conditions. In order that the conditions should be uniform throughout the large number of lines, the following method of culture was found necessary:

As a culture medium, Horlick's malted milk was employed, following the example of Miss Peebles ('12), one-sixteenth of 1 per cent being found the most favorable proportion. This was made fresh each day, with boiling water. The animals were changed every other day. The chief difficulty in making the conditions uniform throughout all the lines is as follows: A number of diverse bacteria are found in the cultures, falling into them at the time of changing, or reaching them in other ways. Some of these multiply strongly in certain of the slide cultures while others get a better foothold in others. The effect of the diverse bacteria on the rate of reproduction differs greatly; as a result therefore some of our lines of *Paramecium* multiply rapidly, others slowly, even though there is no intrinsic differentiation among them. Now, in transferring with a capillary pipette a single individual to a new drop, as is done at the time of changing the animals, inevitably a certain amount of the bacterial culture is transferred with them, serving to infect the new drop. Thus one line will be accompanied always by the bacterium *x*, causing rapid multiplication; another by the bacterium *y*, causing slow multiplication. The results simulate those of inherited differentiation in the fission rate.

Experience showed that this difficulty is obviated by the following method of procedure, which was adopted for the present experiment on August 28:

The new fluid (1/16 per cent malted milk) was made sterile by boiling. It was then infected with bacteria from a mass culture of the race *E*, in which the animals were flourishing strongly. This was done by filtering (through two thicknesses of filter paper) a quantity of the fluid from this culture (in order to remove the *Paramecia*). I added four pipettes full of this filtered fluid to 100 cc. of the fresh culture fluid.

For changing the animals to this, two fresh slides are prepared, each containing three drops of this fresh fluid. A vessel of boiling water is at hand; also a supplementary vessel of the fresh culture fluid. The capillary pipette is first dipped in boiling water, then into the fresh culture fluid, then a single individual is removed with it from the old slide to the first new slide. The pipette is then again disinfected in boiling water and washed in the supplementary dish of culture fluid. Meanwhile, the removed *Paramecium* has been swimming about violently in the three drops of fresh culture fluid, thus washing itself largely free from the bacteria introduced with it. Now, with the cleaned pipette, it is retransferred from this wash water to the second slide of fresh fluid. (In much of my work I gave each animal a second washing in the same way.)

A new 'wash slide' is then prepared, the pipette is disinfected and washed as before, and we proceed to transfer in the same way an individual from the second slide to the wash water and then to its definitive slide. After every transfer the pipette must be disinfected and washed, and new wash water must be used for every individual transferred.

Experience shows that all the details of this painful process are quite necessary if the conditions are to be kept uniform in a large number of lines. Carrying this out for some 250 lines for nearly a month I found so exhausting as to make it practically impossible to continue the experiment for a longer period.

Records. The records of the conjugant and non-conjugant lines for this experiment, conducted in the manner just described, are given in tables 34 and 35 (Appendix). The results of this experiment are of so fundamental an importance for the subject with which the present series of papers deals that I feel it necessary to give the records in detail, showing the number of fissions that occurred in each period of two days. These records will be used farther in studies on inheritance, to follow the present paper.

Explanation of tables 34 and 35. These tables give, for the conjugants and non-conjugants, respectively, of the pure strain *E*, the records of fissions for each line for the entire period (twenty-four days for the

conjugants; twenty-one days for the non-conjugants). The records given are the numbers of fissions that have occurred during the two days ending on the date at the head of the column. (In only one case, for the non-conjugants, in the column headed November 6, is the elapsed period three days instead of two.)

Each pair or split pair consisted of the two mated individuals *a* and *b*. From each *a* and *b* the two sister lines *x* and *y* were kept in progress. Thus from each pair there were derived four lines, *ax*, *ay*, *bx* and *by*. But the lines from both *a* and *b* were kept throughout the experiment in but few cases (16 in the pairs, 22 in the split pairs).

The lines from *a* and *b* were kept in separate moist chambers and changed at different times, so that there is no opportunity for resemblance between them to arise through special similarity of treatment. The two lines *x* and *y*, from a single individual, were however kept in the two concavities of the same slide, in the same moist chamber, and changed in succession. (This was for convenience in replacing one from the other, but in repeating such an experiment, *x* and *y* should be kept in separate moist chambers and handled separately; otherwise the significance of any correlation between *x* and *y* is not entirely clear.)

In working out constants of variation, the period August 27 to September 6 (twelve days) was considered the 'first half' for the conjugants; August 28 to September 6 (eleven days) for the non-conjugants. The second half for the conjugants included twelve days (September 8 to 18); for the non-conjugants, ten days (September 8 to 16).

The blanks left in the column under certain dates indicate that the line in question died out on that date, and *its place was supplied by taking an individual from the sister line x or y, derived from the same parent, that is, from the same a (or b, as the case may be).* But a blank in the final column of totals indicates only that the line in question did not live independently throughout the experiment, but was supplied from its sister line at some date, indicated as just set forth.

In determining mean, standard deviation or coefficient of correlation for any period or periods, only lines that lived independently throughout that period are included. However, for the entire period, the few totals included in parenthesis, in the last column, *are employed* also, since the lines for which they stand coincided with another for only two or three fissions at the beginning.

In working out coefficients of correlation, for successive periods, it is of course necessary to correlate any line with its real ancestral line, and to do this it is necessary to pay careful attention to the blanks left in certain columns and the replacement of certain lines which they indicate. Thus, if in table 34 we wish to correlate the fissions in the first half of the entire period with those in the second half, then when, for example, we enter the fissions for the second half of the time (September 8 to 18) in line 8 *by*, we see that this second half descended partly from 8 *bx*; there is no difficulty, however, in determining exactly how many fissions occurred in the first half. We take in this case for the first half of the period the sum of the fissions for 8 *bx* to September 2, plus those for 8 *by*

for September 4 and 6 (that is, $0 + 2 + 2 + 1 + 3 + 3 = 11$); for the second half, 8 by, September 8 to 16 ($=17$); and similarly for all analogous cases. Thus in the correlation tables a given preceding period may sometimes be counted twice, since it gives rise to two lines of progeny, and is therefore correlated with both. This of course introduces no error into the coefficient of correlation. The other constants (given in table 23) were computed with each period counted but once.

Results. Comparative examination of tables 34 and 35 shows a very great difference between the progeny of the pairs, and those of the split pairs, in respect to variation and differentiation in the rate of fission. This is well shown by observing the range of variation in the two cases. In the split pairs the slowest lines show in twenty-one days 18 fissions, the fastest, 28 fissions. In the pairs the range is (for twenty-four days), from 10 to 35. If we reduce these latter numbers by one-eighth, in order that they may compare directly with those for the split pairs, the range becomes for the pairs 8.75 to 30.6, as compared with 18 to 28 for the split pairs. From September 8 to 16 the range for those derived from the pairs is 1 to 17; for those from the split pairs, 8 to 15.

Working out the mean number of fissions, with the standard deviation and the coefficient of variation for the pairs and split pairs during a number of different periods, we obtain the results shown in table 23. Here the data are given for the first and second halves of the experiment; also for the entire period. In order to have certain periods which are absolutely identical in every respect for the two sets, I give also for the pairs the data for the ten days extending from September 6 to September 16, this period coinciding with the 'second half' for split pairs.

As the last columns of table 23 show, the variability in fission of the lines descended from conjugants was, for the entire period, four times as great as that for those descended from non-conjugants. In the first half of the time it was about twice as great; in the second half five times as great.

Examining tables 34 and 35 to discover the cause of this great difference in variation, we find that the descendants of those that have conjugated are differentiated into a number of distinct lines, with different rates of fission. This will at once be evident

if one compares, among the pairs (table 34) line 1 with line 6, and the latter again with line 4. Line 1 *ax* shows a total during the twenty-four days of 33 fissions, line 6 *ax* of 16 fissions; line 4 *ax* of 13 fissions. Corresponding differences are shown in the other divisions of lines 1, 6 and 4, the differences extending *even to lines descended from the two mates of a pair*. Thus line 1 *ax* has 33 fissions; line 1 *bx*, descended from its mate, 31 fissions; line 6 *ax* has 16 fissions; line 6 *by*, descended from its mate, 17 fissions; line 4 *ax* has 13 fissions; line 4 *bx*, descended from its mate, has 12. If we compare similarly the two lines *x* and *y*, derived from a single member of a pair, we find that their fission rates are close together, while lines derived from different pairs differ greatly.

Certain peculiarities of the fission rate are evident. During the first five or six days after the beginning of fission the different lines descended from the conjugants are more nearly uniform in their rate. Then a number of the lines, such as those belonging to pairs 4, 5, 15 and 26, show a marked decrease in the fission

TABLE 23

Experiment 15; Pure strain E. Constants of variation in fission, for the lines descended from conjugants (pairs), and for those descended from non-conjugants (split pairs), for certain periods of time. The total time is, for the pairs, 24 days, for the split pairs, 21 days. The first half includes for the pairs the twelve days, August 27 to September 6; for the split pairs eleven days, August 28 to September 6; the second half, September 8 to 18 (pairs), September 8 to 16 (split pairs).

	NUMBER OF LINES		MEAN NUMBER OF FISSIONS		DAILY RATE	
	Pairs	Split pairs	Pairs	Split pairs	Pairs	Split pairs
Total time.....	69	145	26.333 \pm 0.613	24.034 \pm 0.096	1.097	1.144
First half.....	78	158	12.154 \pm 0.081	11.424 \pm 0.072	1.013	1.039
Second half.....	83	171	14.241 \pm 0.444	12.614 \pm 0.056	1.187	1.261
September 8-16.....	83	171	12.060 \pm 0.367	12.614 \pm 0.056	1.206	1.261

	STANDARD DEVIATIONS		COEFFICIENT OF VARIATION	
	Pairs	Split pairs	Pairs	Split pairs
Total time.....	7.544 \pm 0.433	1.712 \pm 0.063	28.650 \pm 1.734	7.122 \pm 0.284
First half.....	2.370 \pm 0.128	1.347 \pm 0.051	19.501 \pm 1.065	11.789 \pm 0.453
Second half.....	6.003 \pm 0.314	1.083 \pm 0.039	42.154 \pm 2.885	8.585 \pm 0.315
September 8-16..	4.954 \pm 0.259	1.083 \pm 0.039	41.074 \pm 2.485	8.585 \pm 0.315

rate, which persists throughout the remainder of the experiment. In other lines, such as those derived from pairs 1, 2, 3, 7, 12, the rate remains high throughout the entire period.

The inherited differences between the lines will perhaps be best brought out if we divide the twenty-four days of the experiment into four periods of six days each, and give the number of fissions for each of these periods, for a number of diverse lines. We shall re-group these lines in such a way as to bring out strongly the diversities. The results are shown in table 24. It is observable, for example, that in every one of these four periods the line 3 *ax* has a greater rate of fission than 6 *ax*; similarly 3 *ay* and 3 *bx* show in every period a greater rate than 6 *ay* and 6 *bx*. Comparison of other lines shows the same relations.

TABLE 24

Experiment 15. Pure strain E. Fissions in certain of the conjugant lines, for four successive periods of six days each, so arranged as to exhibit the differences between the lines.

LINE	AUGUST 27-31	SEPTEMBER 2-6	SEPTEMBER 8-12	SEPTEMBER 14-18
3 <i>ax</i>	6	10	9	9
6 <i>ax</i>	3	3	5	5
4 <i>ax</i>	7	4	1	1
3 <i>ay</i>	6	9	9	10
6 <i>ay</i>	3	3	5	5
4 <i>ay</i>	(7)	4	1	0
3 <i>bx</i>	6	9	9	8
6 <i>bx</i>	3	5	6	(5)
4 <i>bx</i>	6	5	1	0
9 <i>bx</i>	6	7	10	9
5 <i>bx</i>	4	5	1	0
1 <i>bx</i>	4	9	10	8
15 <i>by</i>	7	5	2	1
20 <i>by</i>	6	8	11	9
27 <i>by</i>	5	5	5	6
26 <i>bx</i>	6	5	3	1

In some cases a line begins with a high rate of fission, then runs down to a very low one, dividing but once or not at all during the last six-day period. Such is the case, for example, in the lines derived from pair 4. In such cases careful and extended tests were made to determine whether the slow fission rate was characteristic of all the members of the given line. Thus, of line 4 *ax*, seven sets; of 4 *ay*, nine, and of 4 *bx*, eight sets; were kept in progress during the last twelve days of the experiment; all showed the same extremely low rate of fission characteristic for the lines derived from pair 4 in tables 34 and 24.

In the same way, eleven sets of No. 5 *a*, eight of No. 5 *b*, twenty sets of No. 6 *b*, six sets of No. 26 *a*, and eight sets of No. 15 *b* were kept in progress during the last twelve days of the experiment; all of them showed slow rates of fission corresponding closely to those given for the lines in question in table 34.

Of the rapid lines, No. 9 *a* was tested by keeping eighteen parallel lines in progress during the last twelve days of the experiment. All divided rapidly, giving 18 to 20 fissions during the twelve days.

It is thus clear that *the lines descended from the ex-conjugants are differentiated in their inherited characteristics*, some having a rapid rate of fission, some a slow rate, and some an intermediate one (although all were kept under absolutely identical conditions). One result of this inherited differentiation is the production of the very high coefficients of variation shown in table 23.

Are there likewise inherited differentiations among the lines derived from the non-conjugants—the members of the split pairs? Examination of the coefficients of variation in table 23, as well as a general inspection of table 35, shows at once that if there is any such differentiation, it is very slight compared with that among the descendants of the conjugants. If we compare very carefully the records of the different lines in table 35, we find a few cases in which it is doubtful whether there may not be inherited differentiation. Line 3 *ax*, for example, shows a rate of fission somewhat below that of most others, while 6 *by*, 22 *ax*, 40 *ay* and 44 *ax* show rates rather above the average. But the differences between even the extreme cases are very small com-

pared with those between the diverse lines derived from the conjugants. Furthermore, taking the most extreme case of line 3 *ax*, with but 18 fissions, we find that the sister line, 3 *ay*, derived from the same parent, does not show a low rate of fission; so that the slow rate is not characteristic of this entire line. In the conjugant lines, on the other hand, the rates of the two or more sets derived from a single individual we found to correspond closely, showing that the characteristic is an inherited one. It would then appear on the whole probable that all the differences seen among the lines derived from the non-conjugants are simply the slight fluctuations unavoidable where a large number of lines are cultivated.

The question may be tested for both sets in another way. If the differences between different lines are matters of inherited differentiation, then of course lines having a fast or a slow rate in one part of the period of the experiment should have a corresponding rate in the other parts. That is, the rates of fission for earlier and later periods should be correlated. We may therefore determine the coefficients of correlation for successive periods, in both the conjugants and non-conjugants; this will tell us whether the rates of fission are, as a rule, inherited in the different lines.

I have worked out for both sets the correlation between the numbers of fissions in each line (1) in the first half of the experiment compared with the second half; (2) in the second third (September 3 to 8 or 4 to 10) compared with the last third (September 10 to 16 or 12 to 18). This latter comparison was made owing to the fact that the direct physiological effect of conjugation appears to obscure the characteristic differentiations, for some days after conjugation.

Furthermore, I have worked out, for the entire period of the experiment, the correlation between the sister lines, *x* and *y*, derived originally from a single member (of a pair or split pair). If the differences in rate of fission are inherited, these two sister lines should of course be similar, giving a positive coefficient of correlation. The correlation, for both conjugants and non-conjugants, is given in table 25.

As table 25 shows, the lines derived from conjugants give an extremely high correlation. In other words the fact that we are here dealing with lines differentiated in inherited characters is demonstrated by this method as well as by the other evidence. After passing the disturbance due to the direct physiological effect of conjugation, the correlation between successive periods rises to 0.8957 (practically to 0.9), an extraordinarily high coefficient. The correlation between the sister lines x and y is likewise 0.9, showing an almost perfect correspondence.

In the lines descended from the non-conjugants, on the other hand, there is no correlation between the numbers of fissions in successive periods, the coefficients being practically 0. That is, so far as this method can show, *the diversities in fission rate are not inherited*, among the members of a pure race which have not conjugated.

On the other hand, the numbers of fissions for the sister lines x and y , do give a small coefficient of correlation (0.2119).

TABLE 25¹

Experiment 15. Pure strain E. Coefficients of correlation in number of fissions, for successive periods, and for the sister lines x and y , in the descendants of conjugants (pairs) and of non-conjugants (split pairs).

	PAIRS		SPLIT PAIRS	
	Number of lines	Coefficient	Number of lines	Coefficient
First half (10-12 days) with second (10-12 days)	82	0.5743 \pm .050	174	.0120 \pm .051
Second third (6-8 days) with last third.....	81	0.8957 \pm .015	172	-.0020 \pm .051
Total time, x with y	50	0.9017 \pm .018	58	.2119 \pm .085

¹For the pairs the 'first half' comprises the 12 days August 27-September 6 of table 34; the 'second half' the remaining 12 days. For the split pairs the 'first half' is 11 days, August 28-September 6; the 'second half' is the remaining 10 days. The 'second third' comprises September 4-10 (pairs) or September 3-8 (split pairs); the 'last third,' September 12-18 (pairs), or 10-16 (split pairs). The total time is, for the pairs, 24 days; for the split pairs, 21 days. The correlation tables for successive periods are formed by taking the number of fissions of a given line in an early period and entering this on the table in connection with the number of fissions for the same line in the later period.

Whether any significance is to be attached to this is doubtful, since the value of the coefficient is but two-and-a-half times its probable error; and a coefficient of this amount would occur once in ten times as a result of chance distribution. Further, the two sister lines *x* and *y* were kept in the two concavities of the same slide, and one was changed immediately after the other. The result of this may have been to keep the two under slightly more uniform conditions than prevails for two individuals in different moist chambers, giving rise to the slight correlation. The matter will be investigated farther, but in any case it is clear that any differentiation that may exist between the non-conjugant lines is extremely slight; so that correlating the fissions of successive periods gives no trace of it.

The present experiment therefore clears up the difficulty left by the results of Experiment 13. In that experiment, as shown in table 33, the 'non-conjugants' exhibited inherited differentiations, as did the conjugants. It seemed practically certain however that these 'non-conjugants' had gone through previous conjugations, so that the observed heritable differentiations were probably due to these previous conjugations. On page 337, I pointed out the necessity for an experiment in which this matter should be controlled. Our present experiment supplies this need; we know that our non-conjugants here have not conjugated since they came from a single parent individual. And our results show that the inherited differentiations in Experiment 13 were indeed due to conjugation; they do not appear when we deal with actual non-conjugants (lines which have not conjugated since they were all derived from a single individual).

Even if it should turn out that the slight correlation shown by *x* and *y* in the non-conjugants of the present experiment is due to real differentiations between the lines, this result would not modify our present conclusion in any essential way, since the differentiation so indicated would be so slight as to be of quite a different order from that produced by conjugation, the latter giving rise, as we have seen, to coefficients as high as 0.9. Even a slight differentiation arising during vegetative reproduction would be of the highest interest, but it would not alter the positive fact of the

immediate production of strongly marked heritable differences by conjugation.

The data of our present experiment, given in tables 34 and 35, bring to light many other important relations, which will be dealt with in subsequent papers. For our present purposes it is sufficient that the experiment demonstrates that conjugation produces within a pure race heritable differentiations; so that as a result races diverse in their heritable characters arise from a single race with uniform heritable characters.

Our previous experiments had shown that conjugation increases variation; and that the variations observed to follow conjugation are heritable. The present experiment puts the finishing touch on this demonstration by showing that these heritable variations do not arise without conjugation.³ Thus we find that one method of producing new strains is by conjugation.

We have now in hand the essential facts for drawing conclusions as to the actual effects of conjugation on the stock.

IV. RÉSUMÉ OF RESULTS: DISCUSSION, AND CONCLUSIONS

In the foregoing sections are detailed the results of a large number of experiments in which conjugants were compared with non-conjugants of the same stock and the same cultural history. What effects do we find conjugation to produce?

The prevailing view as to the effects of conjugation is that it produces rejuvenescence in the stock. This view is excellently stated in Calkins' recent *Protozoology* ('09), particularly in chapter III. The essentials are somewhat as follows:

If we could take such an entire succession of cells thus formed from the repeated divisions of a fertilized protozoön, and if at any given period could combine them in one mass of cells, we should have the analogue of a metazoön and would find that the protoplasm represented by the aggregate of cells would manifest the same successive periods of vitality as those of youth, adolescence, and old age in Metazoa. We would find that the young cells divided more rapidly than they do later in the cycle; we should find that after a certain time they become sexually mature and are able to conjugate and so to perpetuate the

³ There remains the possibility that heritable variations of a totally different (lesser) order of magnitude may arise during vegetative reproduction.

race; and we would find that, ultimately, evidences of weakened vitality and degeneration appear in the aggregate of cells, and that they finally die of old age (p. 103).

It is conjugation that reinvigorates the stock; for succinct, explicit statements of this we may quote from other papers of Calkins and his associates:

Conjugation between two cells results in the complete reinvigoration of all activities, both physiological and germinal (Calkins and Cull '07, page 376). As with the fertilized egg of a metazoön, the copula or fertilized egg of a protozoön is endowed with a great power of cell reproduction and with a high potential of vitality, and this is the main characteristic of the first period of the life cycle (Calkins '06, page 233). As with the metazoön so with the aggregate of protozoa cells, we note a period of youth characterized by active cell proliferation; this in both groups of organisms is followed by the gradual loss of the division energy accompanied by morphological changes in type of the cells preliminary to conjugation and fertilization and to the renewal of vitality by this means (Calkins '06, p. 232).

The experiments described in the present paper constitute an examination as to how far conjugation actually exhibits these effects in *Paramecium*; as well as how far it shows other results. We shall here summarize and discuss the evidence as to the effects of conjugation on the rate of reproduction; on the vigor or vitality, as evidenced by the comparative mortality; on abnormalities; on its production of variation; on inheritance; and the relation of the results as a whole to the theory of rejuvenescence.

EFFECT OF CONJUGATION ON RATE OF REPRODUCTION

Practically all the experiments show that the average rate of reproduction is less after conjugation than before. That is, if we take two sets of animals of the same stock and history, both ready to conjugate; permit one set to conjugate, and prevent the other, we find that those which have conjugated divide thereafter on the average less rapidly than the others.

In most cases the rate of fission was very considerably greater in those that had not conjugated, the excess usually varying from 25 per cent up to 80 per cent or more. In some cases, however,

the difference is very slight. In no case did the conjugants have a higher rate of fission, although in Experiments 4, 7, 8, 12 and 14 the difference between conjugants and non-conjugants was so small as to be without significance. But in the majority of the experiments, and particularly those which included many cases and were little disturbed by extrinsic factors, those that had not conjugated showed a fission rate higher in a marked degree. And this higher fission rate of the non-conjugants persisted for weeks and months (see the results of Experiments 1, 2 and 6).

So much has been said of the greater reproductive power, the "active cell proliferation," et cetera, of the period following conjugation, that this result appears surprising. Yet those investigators who have examined the matter with the greatest care, came long ago to the same result. Maupas insists again and again, at great length, in opposition to the prevailing views, that conjugation does not increase the rate of reproduction. Since the matter is an important one, and one on which incorrect ideas are prevalent, and since Maupas had evidently done much careful work on the question, it may be worth while to give a résumé of the points he makes. The following passage might well be designed as a statement of the present condition of affairs:

On a affirmé que la faculté fissipare des Ciliés était modifiée par la conjugaison, et que cet acte sexuel avait, pour principal effet, de la renforcer et de l'accélérer. Les Ciliés, au sortir de la conjugaison, se multiplieraient beaucoup plus rapidement qu'ils ne le font plus tard. Cette opinion est devenue courante, et on la trouve reproduite dans les Mémoires et les Traités Généraux, comme une vérité définitivement acquise. Elle a été émise pour la première fois, par Bütschli en 1876, et reprise ensuite par Balbiani, en 1882, qui s'en est emparé, et a même cru en avoir fourni la démonstration expérimentale ('88, pages 254-255):

Maupas then examines the supposed evidence of Bütschli and Balbiani, showing that it amounts to nothing. He sets forth that in his own records of fissions, beginning in a number of cases with ex-conjugants, there is no indication of a greater rate of fission in the early part of the cycle. He says of the fissions:

Elles se succèdent avec une marche uniforme, modifiée uniquement par les variations de température. Je ne me suis pas contenté de cette

unique expérience. J'ai isolé d'autres ex-conjugués de la *Stylonychia pustulata*, puis de l'*Onychodromus grandis*, de l'*Euplotes patella*, du *Paramecium aurelia* et de la *Leucophrys patula*. J'ai suivi, jour par jour, les générations successives de leur descendants, pendant des durées de temps qui ont varié depuis quinze jours jusqu'à un ou deux mois. Chez aucune de ces espèces je n'ai constaté la moindre différence dans la succession de bipartitions. Anciennement ou nouvellement conjugués, tous les individus se sont comportés de la même façon. ('88, pages 255-256).¹

In the paper of 1889 Maupas details experiments with *Paramecium aurelia* (p. 227), *Colpidium colpoda* (p. 247), *Leucophrys patula* (p. 261), *Onychodromus grandis* (p. 321), *Stylonychia pustulata* (p. 329), and *Euplotes patella* (p. 353), all showing that after conjugation these animals do not reproduce more rapidly than later in the history of the strain. In *Onychodromus* and *Stylonychia*, indeed, Maupas found that those which had recently conjugated multiplied more slowly, but he believed this to be due merely to individual variations, and to have no connection with conjugation. He sums up, in opposition to Bütschli, as follows:

J'ai affirmé, en outre, que cette puissance de multiplication se maintient régulière et égale pendant le cycle entier, sans qu'il se produise un affaiblissement graduel depuis la première génération post-syzygienne, jusqu'au retour d'une nouvelle période de maturité karyogamique. Autrement dit, je nie que les Infusoires, au sortir de la conjugaison, jouissent d'un faculté de reproduction plus énergique que plus tard ('89, p. 504).

Richard Hertwig ('89) came, through an experimental study, to similar conclusions, save that he discovered the fact that animals which have conjugated actually reproduce more slowly than those which have not. He was apparently the first to perform the experiment employed on a large scale in the present paper, of separating pairs before conjugation was completed, and comparing these members of split pairs with specimens that had finished conjugating. He gives only a general account of his

¹ Maupas notes that of course in the last stages of morphological degeneration just before death, there is a cessation of fission; but when this condition is reached *rajeunescence is no longer possible*. "Je suis convaincu que si, dans les générations d'un cycle, il se produit un ralentissement, celui-ci se fait sentir seulement dans la période effective de dégénérescence sénile; c'est à-dire, lorsque les Infusoires sont devenus incapable de rajeunissement karyogamique" ('89, p. 504).

experiment, not even mentioning the number of cases examined; but some cultures obtained from the split pairs were kept as long as three months:

Als erstes Resultat ergab sich mir eine auffallende Fruchtbarkeit der an der Conjugation verhinderten Thiere; obwohl ich meine Versuche noch nicht abgeschlossen habe, so möchte ich jetzt schon hervorheben, dass die künstlich getrennten Thiere lange Zeit über sich energischer theilten als Paramaecien, welche die Conjugation durchgemacht hatten ('89, p. 223).

These observations led Hertwig to endeavor to save the theory of rejuvenescence through conjugation, by holding that lack of conjugation results in a rate of fission so great as to be harmful; conjugation would then rejuvenate by slowing and regulating this immoderate rate of reproduction ('89, p. 226).

But the facts appear to be clear, so far as the infusoria go. In view of the large number of experiments made by Maupas on this point, the absolute agreement of his results with those of Richard Hertwig; the fact that these men are perhaps the most thorough investigators that have ever worked along these lines; the further fact that there exist no careful experimental results opposed to these; and finally, the very large body of evidence presented in the present paper, all giving the same results—is it not time that the statements or implications that in the infusoria conjugation results in increased reproduction should disappear from the literature of science?

EFFECT OF CONJUGATION ON MORTALITY

The experiments show that as a rule mortality is much higher, under the same conditions, among those that have conjugated than among those that have been prevented from conjugation. This is true both for conjugation among unrelated individuals, and for that among individuals belonging to the same pure strain.

Accidental influences increasing the death rate quite without relation to conjugation are so numerous, especially in experiments carried on under unfavorable conditions, that here the principle is particularly important that one extensive experiment carried through under ideal conditions, without extrinsic disturbing

factors, gives a truer insight than many imperfect experiments. Such a model experiment is, for present purposes, Experiment 1. In this experiment none of the lines descended from non-conjugants (split pairs) died out during the five weeks of the experiment. Of the lines descended from conjugants, though kept under exactly the same conditions, 38 per cent died out during the same period. In the other experiments some of both sets died, though as a rule with more deaths among the lines derived from conjugants.

In two out of the ten or twelve experiments in which this matter was tested the usual relation was reversed; in both these experiments we are dealing with exceptional conditions. In Experiment 3 the temperature was abnormally high, standing much of the time above 32°C. I have found by long experience in Baltimore that it is not possible to carry on slide cultures of *Paramecium* at such a temperature; from whatever source, the animals rapidly die out. Thus, in this experiment the conditions were so bad that a large proportion of both conjugants and non-conjugants died within the four days of the experiment. But under these conditions the lines descended from non-conjugants died out still more rapidly than those descended from conjugants. Of the former 68.6 per cent died in the four days; of the latter, but 23.4 per cent. The difference seemed clearly due to the furious rapidity at which the non-conjugants multiplied, while the conjugants (as is the rule after conjugation) divided but slowly. There is little doubt but that under usual temperature conditions the advantage would have been, in this case also, with the non-conjugants. The fact, however, that conjugation may be physiologically advantageous under very exceptional conditions is an important one.

* The other case in which the advantage was with the conjugants is Experiment 12. Here we are dealing with a much depressed stock, in which reproduction is slow and mortality high before conjugation. Such a stock can hardly be induced to conjugate; so that but three pairs could be obtained from it. With the six lines derived from these were compared ten lines derived from non-conjugants of the same culture. It is important to note that the latter were not split pairs; in other words, they were not

ready to conjugate (as were the non-conjugants in most of our experiments). Such split pairs could not be had in the present case. Of the six conjugant lines, four (or 66 per cent) died out; but of the non-conjugant lines *all* died.

As set forth in the account of this experiment, the ground for this difference seems to lie in the fact that a certain vigor and power of multiplication are a prerequisite for conjugation; so that in the only three pairs that conjugated are included the only vigorous members of the stock; the others died for the same reason that they did not conjugate.

What are the grounds for the greater mortality of the conjugants, found in the great majority of cases? Two possible grounds occur to one:

1. Conjugation involves extremely complex and delicate cytological processes. It seems possible that these processes are easily diverted into abnormal courses, resulting in abnormalities and death.

2. Conjugation, like fertilization, is a process of uniting diverse germ plasms; of producing new combinations of germ plasm (evidence bearing directly on this will be given in a paper to follow the present one). Possibly some of these combinations are incompatible; or produce results not fitted for continued existence under the conditions.

EFFECT OF CONJUGATION ON ABNORMALITIES

Throughout the experiments it was observed that frequent abnormalities of all sorts occur among the descendants of the conjugants, while among the descendants of non-conjugants such are relatively rare. The grounds just set forth as possibly accounting for the greater death rate of the conjugants, perhaps play a part also in the production of abnormalities.

EFFECT OF CONJUGATION ON VARIATION

The most striking effect of conjugation that appears in comparing the conjugants and non-conjugants, is the great *increase in variability* in the rate of reproduction. In all of the experiments the conjugants are much more variable in this respect than

are the non-conjugants. It will be well to summarize here the coefficients of variation for conjugants and non-conjugants in certain periods of each experiment. This is done in table 26.

As the coefficient of variation was computed for several different periods in most of the experiments, it hardly appears practicable to bring together in table 26 all the coefficients given in the tables of the body of the paper. I have therefore selected the longer periods, with some typical partial periods.

As table 26 shows, the difference in variability between conjugants and non-conjugants is not a slight one, but is very great. The coefficient of variation averages at least twice as great for the conjugants, and in some of the cases given in table 26 it is three or four times as great. There can be no question but that *conjugation increases greatly the variation in rate of reproduction*, both in wild cultures and in pure races.

If in place of studying the variation relative to the mean rate, as shown by the coefficient of variation, we examine the absolute amount of the variation, as shown by the standard deviation, we

TABLE 26

Comparative variability, as measured by the coefficient of variation, for the lines descended from conjugants and for those descended from non-conjugants, in numbers of fissions to a given period; for various experiments of the present paper.

EXPERIMENT	TIME	CONJUGANTS (PAIRS)		NON-CONJUGANTS (SPLIT PAIRS)	
		Num- ber of lines	Coefficient	Num- ber of lines	Coefficient
(Wild Cultures)					
1	First 2 weeks.....	56	53.103 \pm 4.232	59	12.975 \pm 0.819
1	Second 2 weeks.....	42	42.870 \pm 3.689	59	27.743 \pm 1.850
2	First 2 weeks.....	34	32.011 \pm 2.874	51	21.350 \pm 1.489
2	Second 2 weeks.....	19	46.944 \pm 6.196	26	22.847 \pm 2.246
3	Four days.....	36	29.369 \pm 2.528	16	12.756 \pm 1.546
(Pure strains)					
4	Twenty days.....	17	30.828 \pm 3.890	18	19.792 \pm 2.310
5	Six days.....	17	42.782 \pm 5.781	20	14.382 \pm 1.565
13a	Seven days.....	58	40.350 \pm 2.910	82	17.550 \pm 0.953
13b	18 Days, December 8-26.....	14	43.893 \pm 5.976	16	18.263 \pm 2.405
13b	January 2-14.....	14	16.878 \pm 2.069	16	12.832 \pm 1.662
13b	January 30-February 12.....	12	37.899 \pm 5.478	14	19.735 \pm 2.821
13b	December 8-January 29.....	16	21.675 \pm 2.715	14	16.428 \pm 2.150
15	First 12 (11) days.....	78	19.501 \pm 1.065	158	11.780 \pm 0.453
15	Later September 8-16.....	83	41.074 \pm 2.485	171	8.585 \pm 0.315
15	24 (21) days.....	69	28.650 \pm 1.734	145	7.122 \pm 0.284

shall come to the same result, finding that in every case the variations are not only relatively, but absolutely, greater among the conjugants. The standard deviations corresponding to the coefficients of variation given in table 26 will be found in the tables included in the body of the paper, under the different experiments.

In just what way is the variation increased in the conjugants? That is, do we find that after conjugation there are more specimens with a lower rate of fission, or with a higher rate of fission, or with both? What is the nature of the distribution of the fission rates in each case?

The fact that the mean rate is lower for the conjugants would cause us to suspect that the increase in variation is at least partly due to a decrease in the rate of fission of some of the lines, while others remain high. Examinations of the data shows that this is largely true. To bring out this point, it will be well to note the comparative range of variation in number of fissions, for the conjugants and non-conjugants, in the various experiments. This is exhibited in table 27. In this table are included the number of fissions for only the lines that lived through the period specified.

As table 27 shows, at the lower extremity the conjugant lines range much farther than the non-conjugant lines; in every case the lower extreme for the conjugants is below that for the non-conjugants, and in many cases the difference is very considerable. At the other extremity of the range no such great difference is found. The maximum is, as a rule, higher for the non-conjugants, but this is not invariable; in some cases the maximum for the conjugants is equal to that for the non-conjugants; or even a little greater.

It appears therefore that conjugation increases the variation mainly toward the lower extremity of the range; it produces many lines whose rate of fission is lower than that for the non-conjugants, while others remain high. But even in the middle regions of the range, the conjugant lines are less heaped up about the mean than the non-conjugants. These peculiarities may be illustrated by examination of the distribution of the variations in the experiments with larger numbers, as given in table 28, for Experiments

1 and 15. With this table may also be examined tables 9, 10, 11 and 20, which give the same data for various other experiments.

The spreading out toward the lower end of the range in the lines descended from pairs is very striking in table 28. In some cases it appears that the lines descended from conjugants tend to differentiate into two groups, one with a low fission rate, the other with a higher one. This is particularly notable in the data for Experiment 15, in table 28, but is observable also in Experiment 1.

TABLE 27

Comparative range of variation in lines descended from conjugants, and in those descended from non-conjugants, for fissions in a given period.

EXPERIMENT	TIME	CONJUGANT		NON-CONJUGANTS	
		Minimum number of fissions	Maximum number of fissions	Minimum	Maximum
(Wild cultures)					
1	First 2 weeks	0	12	7	15
1	Second 2 weeks	0	16	6	20
1	Total 4 weeks	9	28	13	35
2	First 2 weeks	0	11	7	15
2	Second 2 weeks	0	13	6	11
2	Eight weeks	25	38	37	47
3	Four days	2	10	8	13
(Pure strains)					
4	Twenty days	1	17	6	17
5	Six days	0	6	4	6
7	Nine days	10	14	11	16
9	Sixteen days	19	22	23	27
13a	Seven days	0	10	4	10
13b	December 8-16	4	21	12	21
13b	January 2-14	7	12	8	12
13b	January 30-February 12	3	15	9	14
13b	December 8-January 29	21	46	24	50
15	12 (11) days	6	16	8	15
15	September 8-16	1	17	8	15
15	24 (21) days ¹	{ 10 (8.075)	{ 35 (30.06)	18	28

¹ In the last entry, for experiment 15, the time for the conjugant lines is 24 days, that for the non-conjugants but 21. If we reduce by one-eighth the fissions for the conjugants, they will then be comparable with those for the non-conjugants; this gives the figures shown for this case in parenthesis.

It will be observed from table 28 that even in that part of the range where the non-conjugant lines are found, the conjugant figures are much less heaped up near the mean than are those for the non-conjugants. This shows clearly that the greater variability of the conjugants is not due alone to an extension of the range of variation toward the lower end; but also to a scattering of those lying near the mean. If, for example, we omit in Experiment 15 all the conjugant lines lying lower (in table 28) than any of the non-conjugant lines, we still find the variation for the conjugants to be much greater than that for the non-conjugants. In Experiment 15, making the omission mentioned, the coefficient of variation for the conjugants would be 16.776, as compared with but 8.585 for the non-conjugants.

Conjugation, then, increases variability in reproductive power. The next question is: Are these differences inherited, so that in this way differentiated races are produced? To this question were mainly dedicated Experiments 13 and 15, and, as the account given in the text shows, *the differences thus produced are inherited*. In wild cultures, such as that of Experiment 1, this question cannot be answered so clearly, since the differences in fission rate existing before conjugation are likewise inherited and the effect of conjugation is only to increase the number and extent of these

TABLE 28

Distribution of the number of fissions for the lines of descendants of conjugants, as compared with those from non-conjugants, for certain periods in experiments 1 and 15. (The table shows, for example, that in the first two weeks of experiment 1, three of the conjugant lines did not divide; four divided once, etc.)

		NUMBER OF FISSIONS																	TOTAL FISSIONS	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		17
Experiment 1--																				
First two weeks:																				
Pairs.....		3	4	1	2		1	3	3	6	4	13	11	2	1				56	
Split pairs.....									2		1	2	3	15	18	12	5		59	
Second two weeks:																				
Pairs.....		2		1	2		2	2	2	4	5	7	2	4	2	3	2	2	42	
Split pairs.....								2	3	3	3	6	5	11	4	5	7	3	59	
Experiment 15--																				
September 8-16																				
Pairs.....			6	5	2	1					2	2		2	5	9	13	20	12	83
Split pairs.....											1		6	13	52	68	27	4		171

inherited differences. But working with a pure race, as in Experiments 13 and 15, it is found (1) that differences in rate of fission among those that have not conjugated since they were derived from a single parent are not inherited (unless possibly certain differences of a minimal character are to be excepted; differences of an order of magnitude far below those with which we are dealing); (2) that conjugation among the members of such a pure race does result in differentiations that are inherited, so that from a race homogeneous with respect to fission rate, we get many races, differing in their rates. The hereditary differences thus produced are not small and inconstant but so decided as to give coefficients of correlation up to 0.9 between earlier and later generations, in spite of fluctuations due to environmental differences.

To what is due the production of inherited differentiations by conjugation? Here for the present we can only speculate. It would seem probable that we have before us something of the process that we see in Mendelian inheritance. If the members of a culture differ in their germinal make-up, conjugation among them would produce many new combinations of germinal characteristics. The fact that we find such heritable differentiations produced by conjugation among the members of the same pure race would be accounted for if the members of the race are heterozygotic, although all alike in germinal composition. Interconjugation among such similar heterozygotes would, on Mendelian principles, produce many new combinations of germinal constituents, just as happens in the self-fertilization of higher organisms.

In connection with such a view of the matter, it needs to be recalled, however, that in our Experiment 13 we were dealing with a stock that had gone through eight successive self-fertilizations, the stock being derived, after each of these, from a single ex-conjugant. Such a series of eight self-fertilizations would, as set forth in the account of Experiment 13, go far in getting rid of heterozygosity, unless the character we are studying depends on a very large number of independent factors. In that stock we nevertheless found that inherited differentiations as to fission rate were produced by conjugation. This may indicate that Men-

delian recombination is not the whole secret of the matter; it does not, however, demonstrate this.

In a previous paper ('11), I have shown that conjugation likewise increases variability in size. In the account of Experiment 9, of the present paper, some data are given indicating that the size differentiations so produced are likewise inherited. But the results there given are by no means conclusive, the matter requires further study.

It seems best to reserve for a later paper on inheritance a comparative review of what is known as to the production of variation by conjugation in other organisms, with the various theories that have been held.

CONJUGATION AND BIPARENTAL INHERITANCE

In a paper to be published at once, by the present author and K. S. Lashley, it will be shown that conjugation results in inheritance from both the parents that enter into the pair. All details are reserved for the paper referred to; the matter is mentioned here merely to complete the outline as to effects of conjugation.

CONJUGATION AND THE THEORY OF REJUVENESCENCE

The chief positive results from the present investigation are: (1) that conjugation increases variation, giving rise to heritable differentiations; (2) that it results in biparental inheritance (to be taken up in a separate paper); (3) that the fission rate is lower after conjugation; (4) that the mortality is as a rule higher, and abnormalities are more common, among the descendants of conjugants than among those of non-conjugants.

What is the relation of these results to the theory that conjugation produces rejuvenescence?

A number of diverse things have been included under rejuvenescence, the theory meaning for some authors one combination of these, for others another combination. The main points included appear to be the following:

1. The structural changes—the replacement of the old macronucleus by a new structure derived from the micronuclei—has

sometimes been held to constitute a visible rejuvenescence, a rejuvenescence of the macronucleus. This in some forms is accompanied by a renewal of other structures, as for example, of the bodily appendages in the *Hypotricha*. Engelmann ('76) emphasized these changes as constituting in themselves "ein wahren Verjüngung" (p. 629).

This actual replacement of old structures by new no one will of course deny, and it seems not inappropriate to call it a rejuvenescence, if we mean by this word nothing more than these observed facts.

2. But the theory has as a rule gone far beyond these observed facts. Thus, Maupas says, after a statement of these structural changes:

Ce nouveau appareil nucléaire agit sur tout l'organisme, auquel il appartient, comme une sorte de ferment régénérateur, lui restituant, sous leur forme parfaite et intégrale, toutes les énergies vitales caractéristiques de l'espèce. Cet être se trouve donc rajeuni dans le sens littéral et absolu du mot. Il peut dès lors redevenir le progéniteur d'un nouveau cycle de multiplications agames, dont toutes les générations successives seront douées des mêmes facultés rajeunies, jusqu'à ce que celles-ci s'usent et s'affaiblissent peu à peu, par leur exercice même, et en arrivant ainsi à ressentir le besoin réparateur d'une nouvelle période d'activité fécondatrice ('89, p. 434).

Now, in the passage, we have quoted, Maupas evidently affirms certain things that by no means follow from the structural changes observed, but can only be demonstrated by the results of experimentation. We shall have to inquire how far these have been thus demonstrated. But before doing this, we must proceed to one farther development of the theory.

3. As we have seen (page 357), Maupas did not hold that the vigor and rate of reproduction are increased by conjugation, although such a general statement as the one above quoted would seem to imply that this is true. This idea has, however, been held by many as a fundamental part of the theory of rejuvenescence. The rate of reproduction has been held to become less and less as the number of vegetative generations increases, until by a new conjugation it is brought back again to its original level (see the quotations on page 356).

This part of the theory of rejuvenescence which holds that the vigor of reproduction is increased by conjugation appears to be definitely a mistake, for the infusoria, as we have already shown (page 359). We shall therefore consider it no farther.

The experimental results of the present paper of course do not alter the facts as to the 'structural rejuvenescence' if one desires so to call it. Certain points are worthy of notice in this connection.

1. So far as the 'rejuvenescence' or renewal of structures other than the macronucleus is concerned (locomotor organs, et cetera), this takes place equally in vegetative reproduction. It furnishes therefore no foundation for a theory that conjugation is in any special way a rejuvenating process.

2. The replacement of the macronucleus by parts of the micronuclei of the two individuals of the pair is of course thoroughly in consonance with the results of the present study, furnishing not the slightest difficulty for interpretation. The micronuclei are to be conceived as corresponding to the nuclear apparatus of the germ cells of higher organisms, each one consisting of a certain combination of 'determinants' or 'genes.' When the macronucleus is replaced by parts of two micronuclei, a new combination of 'determinants' is thus produced; the progeny may therefore differ from the parents. In other words, 'variation' is induced in conjugation—through the production of many new combinations, in different cases. Again, since the new macronucleus is produced by the union of parts from two diverse individuals, the progeny may inherit from these two; in other words, conjugation results in biparental inheritance, as we have actually found to be the case.

Now, it is a priori not impossible that the effects of the renewal of the macronucleus are completed in those two results; it is not a priori certain that the new macronucleus must otherwise function any better than the old one.

We must therefore inquire as to the experimental ground for the assertion made in the quotation given above from Maupas, to the effect that this new apparatus acts on the entire organism as a

sort of regenerating ferment, restoring all its vital energies, et cetera.

The grounds for this view have consisted, almost exclusively, not in actual observation of any such rejuvenizing action by conjugation, but in the observation that during vegetative reproduction under experimental conditions the organisms become depressed, degenerate, and finally die. From this it was concluded that conjugation must be what remedies this.

This line of argument has, however, quite lost its force, in view of the modern work of Calkins, Enriques, Woodruff, and others. These authors' results demonstrate that the very limited periods within which Maupas observed degeneration has no significance for the question as to whether degeneration is an inevitable consequence of continued reproduction without conjugation, for they kept vegetative reproduction in progress for periods many times as long as those which Maupas found to result in degeneration. The work of Woodruff, in particular, seems to show that *Paramecium* may be kept multiplying vegetatively for an indefinite period. Furthermore, the work of Enriques and of Woodruff has shown to what the degeneration observed by Maupas was due. Under proper nutritive and chemical conditions no such degeneration appears.

It is not necessary to review in detail this vast subject, but there will hardly be any dissent from the statement that the modern work has largely, if not entirely, deprived of its force this argument for the necessity of conjugation.

All the more therefore we are driven to examine the direct evidence as to the rejuvenating effect of conjugation. And in doing so, we must reflect that if the argument above mentioned were valid, there should be no difficulty in observing experimentally the rejuvenating effect; so that a fortiori we must demand what this direct evidence is.

In reading Maupas' great works ('88, '89) in search of this direct evidence for a rejuvenating effect of conjugation, one is astonished at the way it eludes one at every step. Most of the actual observations that bear on the matter at all, seem indeed

opposed to the rejuvenating action of conjugation. Maupas demonstrated, as we have seen, by extended experimentation, that conjugation is not followed by an increase in the vigor of multiplication. He found, in repeated observations, that conjugation within his degenerating stocks did not help them, but attributed this to their being closely related. But he observed further that *when the depressed stocks that interconjugated were not related, they still died after conjugation*, so that conjugation did not remedy degeneration in the one case or the other ('89, p. 409). He found that conjugation is often sterile (followed by death) in *wild* cultures of *Stylonychia* ('89, p. 331). He found that ex-conjugants of *Spirostomum*, *Climacostomum* and *Didinium* did not reproduce farther ('89, pp. 277, 295, 297). In *Leucophrys* a large proportion of the conjugants die ('89, p. 254-255). He found that in some cases a second conjugation follows a first one after but a few generations (*Leucophrys*, '89, p. 409). He found that animals which are ready to conjugate may be prevented, and they will then continue to multiply with uninterrupted vigor ('89, p. 306). All these observations speak against rather than for the idea of a regular cycle of vegetative reproduction, resulting in degeneration, and requiring conjugation at a certain stage, this remedying the degeneration.

Has Maupas absolutely no evidence that conjugation rejuvenates? He seems possibly to have held that the following fact is evidence of this effect. In his long continued cultures, he found that when the animals derived from a single parent interconjugated, they later died. It is notable that this result has not been confirmed by later investigation, and Maupas himself noted certain exceptions. But Maupas found that when he mixed individuals from different cultures, the pairs were fertile (provided both did not belong to degenerated cultures). It would appear that Maupas supposed that rejuvenescence had taken place in these cases. But of course there is absolutely no evidence that such has occurred, unless it is shown experimentally that the ex-conjugants are more vigorous and propagate longer than similar parents who did not conjugate. In view of the

results given in the present paper, where the reverse is shown to be the rule, it is clear that these observations of Maupas do not touch the matter at all.

One single case only Maupas has which makes even an approach to the form of this necessary demonstration, and this, as we shall see, really gives no evidence at all. This is the case of one of his cultures of *Stylonychia pustulata* ('88, pp. 196-201). A line of propagation was begun with a single individual, November 1, 1885. This line died out on March 26, 1886, after 215 generations. On February 22 a single specimen of the 156th generation was taken from this line and allowed to conjugate with an individual from outside. Maupas tells us on page 323 of his paper of '89 that these individuals from outside, which he mixed with those from the long-continued cultures "were taken at hazard in my small aquaria." Thus such an individual had not been living under the peculiar conditions of these experiments. Derived from this pair a new line of propagation was continued for 316 generations (till July 10, 1886), while the old line from which one of these ex-conjugants came, died out after but 59 generations more.

Now, the work of Enriques, Woodruff, Baitsell ('12), et cetera, has shown that the conditions with which Maupas worked result after a time in depression of the vital functions, but that animals kept under more favorable conditions do not show such depression, even though they have lived as long without conjugation as the depressed race. The depression is due to the conditions, not to lack of conjugation. What Maupas did was to take from outside a fresh, vigorous specimen, and mate it with one of these depressed ones. He then found that the progeny were vigorous. *He does not note whether the line of progeny he used came from the depressed member of the pair, or from the vigorous one*, although this is an absolutely essential point for determining whether the depressed stock was rejuvenated even by conjugation with a vigorous one. The probability is strong that the new line of propagation came from the new, vigorous individual. But such an individual would have given an equally long series of vegetative propagations if it had not been mated at the beginning. Its

vigor was due to the fact that it had been living under favorable conditions, not to conjugation.

There is absolutely nothing in this experiment to demonstrate that a partially exhausted race is rejuvenated by conjugation. A real test would be the following: Two unrelated lines should be allowed to multiply till both become depressed. Then they should be allowed to conjugate, to determine whether the conjugation remedies the depression. It will manifestly not do, in testing the question whether conjugation remedies depression, to take a vigorous, undepressed specimen as one member of the pair. According to the cyclical theories, *all* lines of propagation become depressed after a series of vegetative reproductions, so that if conjugation is to maintain the race, it must be effective when it occurs between two lines, both of which are depressed.

Now, as we have briefly mentioned above, *Maupas performed this crucial experiment*. He kept lines of propagation of *Stylonychia* of diverse origin till they became depressed, then allowed them to conjugate one with another. This fact is briefly set forth on page 409 of his paper of 1889. Such cross-conjugation of two diverse lines *did not result in rejuvenescence*; the animals died just as happened when the two members of a pair came from the same parents. Speaking of sterile conjugations, Maupas says, "Elles s'effectuent, en effet, aussi bien entre individus appartenant à un même cycle ou proches parents, qu'entre individus étrangers l'un à l'autre et provenant de cycles différents" ('89, p. 409).

Anyone who goes critically through the 480 pages of Maupas' two great papers for the purpose of finding out what evidence there is that conjugation rejuvenates, will, I believe, be forced, as I have been, to realize that they contain no evidence for this whatever, although they do contain evidence against it. Maupas' conclusion was evidently due to the supposed theoretical necessity for something to remedy the degeneration induced by long vegetative reproduction under the conditions of his experiments. *All that his experiments show is that long continued propagation under the given conditions results in injury to the stock—and this equally whether there is or is not conjugation within the stocks, or between*

two such stocks of diverse origin. There is thus not even any indirect evidence that conjugation rejuvenates, since the stocks that conjugated underwent the same fate as those that did not.

So far as I have been able to discover, there is no experimental evidence from any other source that conjugation rejuvenates. In Miss Cull's paper entitled "Rejuvenescence as the result of conjugation" ('07), the evidence consists merely in showing that a considerable fraction of those that had conjugated continued thereafter to multiply. But control experiments show, as set forth in the body of the present paper, that they would have continued equally if they had not conjugated; in fact a larger proportion would have continued to multiply if they had not been allowed to conjugate. There is thus in these results no evidence of rejuvenescence through conjugation; and this must be said of all observations which merely show that some of the ex-conjugants continue to multiply. Control experiments with animals prevented from conjugating are necessary for a correct understanding of the results.

In the long series of studies set forth in the present paper as Experiments 5 to 14, the effects of conjugation were studied when one division of a race is allowed to conjugate frequently, while another is kept from conjugating; also the effects of conjugation in a race that is actually depressed. As to the first point, the animals that did not conjugate were found throughout to be more vigorous than those that conjugated frequently.

With regard to the effects of conjugation in a depressed race, it is to be recalled that Maupas had repeatedly tried this experiment, finding always that conjugation has no beneficial effect under such conditions. The question might then be regarded as settled, since there is no expectation of beneficial effect even accepting the views of the great upholder of the theory of rejuvenescence; positive results would be directly opposed to the experimental results of Maupas.

Yet it was in one of these experiments alone that any result was reached that could possibly lend themselves to an attempt to maintain that conjugation has a beneficial effect on vigor and vitality. In Experiment 12 the stock was so depressed that it

multiplied scarcely at all, and the mortality was high. It was almost impossible to get conjugation among its members, since a prerequisite to conjugation is a period of rapid multiplication. The necessary conditions were fulfilled only for three pairs. From these, six ex-conjugants were obtained. The six lines of propagation derived from these were compared with ten lines from individuals that did not conjugate (and did not attempt to do so).

There was no general rejuvenescence due to conjugation. Three of the six conjugant lines died out within a week, and a fourth a little later; so that two-thirds of the conjugant lines were dead. But two continued to multiply. But in the mean time *all of the ten non-conjugant lines died out.*

What has happened here? We can hardly speak of rejuvenescence where two-thirds of the ex-conjugants die out. The survival of some of the conjugants may have been due to the greater vigor that was a prerequisite to their conjugation, the lack of which caused the others not to conjugate. Aside from this we can only say that the results of conjugation were here the same as usual; *it induced variation in the reproductive power.* As always, some lines derived from the conjugants had a low reproductive power and died at once. Two out of the six had greater reproductive power; they therefore continued to multiply. In the meantime, the uniform non-conjugants, retaining the original depressed condition, all died out after a short time.

This experiment therefore gives, in fact, the same result as all the others, an increase of variation as a result of conjugation. It differs from the others merely in the fact that in two of the six cases the extremes of variation reached a higher level than that which characterized the animals before conjugation. The same result is reached in Experiment 15, where the conjugants at the upper extreme of the range exceeded in their rate of reproduction the uniform non-conjugants. But in this latter case there is no temptation to speak of rejuvenescence, since the non-conjugants still continue to multiply vigorously.

Thus, under exceptional conditions the production of variation by conjugation results in preserving some representatives of a stock which would otherwise die out completely.

The results of the present investigation on the effect of conjugation need to be considered in connection with the results of the investigations of Calkins, Enriques, Woodruff, and others, on the results of long continued vegetative reproduction. The two lines of work complement each other and lead to harmonious and definite conclusions. In a recent brief paper ('12 a) I have reviewed the two in their relation to each other. Here I shall not attempt to review the work on vegetative reproduction but merely to summarize the common result of both lines of work.

GENERAL CONCLUSION

Comparing conjugation with the fertilization of higher animals, we find the following to be the state of the case:

In higher animals fertilization has two diverse effects, which recent investigation, particularly that of Loeb and his associates, has clearly disentangled. (1) On the one hand, it initiates development; it prevents the egg from dying, as it would do if not fertilized. This function of fertilization is the one that is replaced by the processes which induce artificial parthenogenesis. (2) But, secondly, fertilization brings about in some way inheritance from two parents. When there is inheritance from but one parent, the inheritance is as it were complete; the child as a rule resembles its parent in all hereditary characteristics; this is the result of the so-called 'pure line' work. But when we have biparental inheritance, a great number of different combinations of the characteristics of the two parents are produced, so that the process of fertilization is one that in this respect completely alters the face of organic nature, producing infinite variety in place of relative uniformity.

These two functions of fertilization, the initiation of development, on the one hand, and the production of inheritance from two parents, on the other, are logically independent; they might conceivably be performed at different times and by different mechanisms. The fact that in many organisms the same mechanism that brings about biparental inheritance is likewise the one that initiates development might from certain points of view be

called an adaptation. Its result is to insure that in *all* organisms that develop there shall be inheritance from two parents, not from one. In the work on artificial parthenogenesis these two functions have been separated experimentally; the initiation of development takes place alone.

Now, in endeavoring to understand conjugation, attention has been given hitherto almost exclusively to the first of these two functions. It was held that the function of conjugation must be to make possible life and development where it was otherwise impossible, just as fertilization arouses the egg to further life and development. But it turns out that in the infusoria conjugation, instead of having this one of the two functions of fertilization, has the other. The two functions are in the infusorian separated, just as they are in artificial parthenogenesis, but it is the second, not the first, that we have before us. Conjugation is not necessary in order that life and reproduction shall continue; they continue without it. There is no evidence that conjugation in the infusoria increases the reproductive power, or rejuvenates the organism physiologically in any way.

But the life which thus continues is uniform and unchanging. To give biparental inheritance, with varying mixtures of the characteristics of the two parents; to produce these new combinations in great variety, conjugation is necessary. And when this happens under such conditions that the original combinations were not adapted to survival, then some of the new combinations produced often are adapted to the conditions; conjugation then results in a survival of an organism that would have been completely destroyed without it. It is most interesting in this connection to observe that conjugation is usually induced by an unfavorable change of conditions, a change of such a nature that the organisms begin to decline. Thereupon conjugation occurs, so that new combinations are produced, adapted to varied conditions, some of which may survive.

Thus the whole series of investigations on vegetative reproduction and on conjugation leads to a unified result, and one that is in consonance with what we observe in higher animals.

Our main results may then be summed up as follows: So far as physiological effects are concerned, conjugation does not produce rejuvenescence, for after conjugation most of the animals are less vigorous than before. *What conjugation does is to bring about new combinations of germ plasm, just as is done in the sexual reproduction of higher animals. One result of this is to produce biparental inheritance; another is to give origin to many variations, in the sense of inherited differentiations between different strains. Some of the new combinations are better adapted to the existing conditions than others; these survive while the others die out.*

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APPENDIX: FUNDAMENTAL TABLES

TABLE 29

Experiment 1. Paramecium caudatum. Number of fissions per week in the 61 lines derived from conjugants, and in the 59 lines derived from those that have not conjugated. May 4 to June 7, 1909. Numbers in parenthesis indicate that the line in question had died out before the end of the experiment. (d = died out, during the week indicated.)

Pairs (conjugation consummated)

PAIR	INDIVIDUAL	WEEK					TOTAL	PAIR	INDIVIDUAL	WEEK					TOTAL	PAIR	INDIVIDUAL	WEEK					TOTAL
		1	2	3	4	5				1	2	3	4	5				1	2	3	4	5	
1	a	0	1	d			(1)	12	a	6	5	3	4	2	20	23	a	4	5	5	2	d	(16)
	b	1	2	d			(3)		b	4	7	7	5	2	25		b	5	6	4	1	2	18
2	a	5	6	8	5	1	25	13	a	5	7	7	5	4	28	24	a	4	6	6	2	2	20
	b	5	5	6	4	1d	(21)		b	1	6	6	3	1	17		b	1	0	d			(1)
3	a	4	6	8	6	2	26	14	a	4	7	6	6	3	26	25	a	4	4	1d			(9)
	b	2	6	1	1	d	(10)		b	4	6	7	3	3	23		b	4	6	4	5	0	19
4	a	1	0	0	d		(1)	15	a	1	0	1	d		(2)	26	a	3	6	5	6	2	22
	b	5	5	5	4	2	21		b	2	5	d			(7)		b	3	7	7	8	5	30
5	a	0	2	0	d		(2)	16	a	3	2d				(5)	27	a	0	d				(0)
	b	5	4	6	4	2	21		b	3	2	3	3	3	14		b	4	4d				(8)
6	a	6	5	6	4	7	28	17	a	5	7	7	2	3	24	28	a	4	4	d			(8)
	b	3	5	3	0	6	17		b	1	d				(1)		b	4	6	3d			(13)
7	a	2	1	3	3	4	13	13	a	5	6	9	7	2	29	29	a	4	7	4	6	5	26
	b	4	2	d			(8)		b	3	5	5	4	1	18		b	4	8	9	5	4	30
8	a	0	6	8	4	3	21	19	a	5	6	0	0	2	13	30	a	3	5	7	6	3	24
	b	4	6	5	5	3	23		b	5	5	0	0	2	12		b	3	6	4	4	3	20
9	a	0	0	d			(0)	20	a	4	2	2	1	1	10	31		0	0	d			(0)
	b	0	0	d			(0)		b	3	4	3	2	2	14								
10	a	4	d				(4)	21	a	5	5	9	5	2	26								
	b	5	6	2	6	3	22		b	5	8	8	7	4	32								
11	a	4	6	7	3	d	(20)	22	a	5	6	4	4	1	20								
	b	4	5	6	5	3	23		b	3	7	9	7	4	30								

TABLE 29 (CONTINUED)
Split pairs (conjugation not consummated)

SPLIT PAIR	INDIVIDUAL	WEEK					TOTAL	SPLIT PAIR	INDIVIDUAL	WEEK					TOTAL	SPLIT PAIR	INDIVIDUAL	WEEK					TOTAL
		1	2	3	4	5				1	2	3	4	5				1	2	3	4	5	
1	a	6	4	7	6	5	28	11	a	8	6	10	7	4	35	21	a	7	6	5	3	4	25
	b	6	6	9	7	3	31		b	8	6	6	5	4	29		b	8	7	6	1	1	23
2	a	8	6	6	6	2	28	12	a	7	6	3	4	1	21	22	a	6	7	7	2	3	25
	b	7	7	9	6	3	32		b	7	7	3	5	2	24		b	7	6	2	4	3	22
3	a	7	6	7	5	2	27	13	a	4	7	8	7	4	30	23	a	8	7	10	7	2	34
	b	7	5	6	6	2	26		b	7	7	7	5	1	27		b	7	7	5	7	3	29
4	a	7	4	6	4	1	22	14	a	7	8	2	4	2	23	24	a	7	7	6	4	3	27
	b	7	6	8	7	5	33		b	5	7	6	5	3	26		b	6	6	5	4	3	24
5	a	7	6	6	6	4	29	15	a	5	7	8	5	4	29	25		7	5	10	5	1	28
	b	8	4	5	5	5	27		b	5	7	3	6	2	23	26		4	3	3	3	1	14
6	a	7	5	9	6	3	30	16	a	6	4	5	3	2	20	27		7	6	8	3	3	27
	b	8	6	10	7	3	34		b	7	6	4	3	3	23	28		9	5	8	8	2	32
7	a	7	5	9	5	0	26	17	a	7	6	7	4	0	24	29		7	6	5	5	4	27
	b	7	6	8	6	1	28		b	7	6	9	6	2	30	30		6	6	5	5	1	25
8	a	7	6	7	5	2	27	18	a	8	6	9	6	1	30	31		8	7	8	6	4	33
	b	7	5	7	5	2	26		b	7	5	8	4	2	26	32		6	7	1	5	3	22
9	a	7	5	9	5	1	27	19	a	6	7	6	7	4	30	33		5	4	4	2	2	17
	b	8	6	8	6	3	31		b	5	8	9	7	3	32	34		3	4	5	5	3	23
10	a	6	6	7	5	2	26	20	a	7	6	7	4	6	30	35		8	6	5	3	2	29
	b	8	7	12	8	3	38		b	6	5	6	4	0	21								

TABLE 30

Experiment 2. *Paramecium caudatum*. April 10 to June 4, 1909. Number of fissions, first two weeks, for those that have conjugated (pairs) and those that have not ('split pairs' and 'free'). (d = died.) Numbers in parenthesis give total fissions for those that died before the end of the period.

Pairs

PAIR	INDIVIDUAL	WEEK		TOTAL	PAIR	INDIVIDUAL	WEEK		TOTAL	PAIR	INDIVIDUAL	WEEK		TOTAL	PAIR	INDIVIDUAL	WEEK		TOTAL
		1	2				1	2				1	2				1	2	
1	a	4	2	8	9	a	2	1	3	17	a	3	1	4	25	a	4	2d	(6)
	b	0	0	0		b	3	2	5		b	6	1d	(7)		b	5	2	7
2	a	6	3	9	10	a	5	1	6	18	a	4	3	7	26	a	d		(0)
	b	6	3	9		b	4	1	5		b	4	3d	(7)		b	3	2d	(5)
3	a	d		(0)	11	a	5	d	(5)	19	a	d		(0)	27	a	1	d	(1)
	b	3	4	7		b	5	2	7		b	3	2	5		b	4	1d	(5)
4	a	3	3	6	12	a	4	2	6	20	a	3	2	5	28	a	6	d	(6)
	b	4	2	6		b	5	2	7		b	4	0	4		b	4	1d	(5)
5	a	4	1	5	13	a	1	d	(1)	21	a	3	1d	(4)	29	a	8	1d	(9)
	b	4	2	6		b	3	1	4		b	0	d	(0)		b	lost		
6	a	4	1	5	14	a	6	2	8	22	a	6	5	11					
	b	3	2d	(5)		b	d		(0)		b	6	2d	(8)					
7	a	3	2	5	15	a	5	2d	(7)	23	a	6	2	8					
	b	d		(0)		b	4	3	7		b	5	4	9					
8	a	1d		(1)	16	a	d		(0)	24	a	5	2	7					
	b	5	2	7		b	5	2	7		b	5	2	7					

TABLE 30 (CONTINUED)

Split pairs

SPLIT PAIR	INDIVIDUAL	WEEK		TOTAL	SPLIT PAIR	INDIVIDUAL	WEEK		TOTAL	SPLIT PAIR	INDIVIDUAL	WEEK		TOTAL	SPLIT PAIR	INDIVIDUAL	WEEK		TOTAL
		1	2				1	2				1	2				1	2	
1	a	6	4	10	6	a	8	4	12	11	a	6	2d	(8)	16	a	8	2	10
	b	8	4	12		b	9	5	14		b	7	1	8		b	8	4	12
2	a	9	2d	(11)	7	a	4	3	7	12	a	6	2	8	17	a	7	2	9
	b	8	2	10		b	7	1d	(8)		b	8	d	(8)		b	7	1d	(8)
3	a	11	4	15	8	a	d		(10)	13	a	7	1d	(8)	18	a	4	d	(4)
	b	6	3	9		b	9	4	13		b	8	2	10		b	4	d	(4)
4	a	7	4	11	9	a	8	d	(8)	14	a	8	d	(8)	19	a	6	2d	(8)
	b	9	1	10		b	7	2d	(9)		b	7	2	9		b	9	d	(9)
5	a	8	3	11	10	a	9	d	(9)	15	a	7	1	8	20	a	8	d	(8)
	b	6d		(6)		b	7	d	(7)		b	8	2	10		b	lost		

Free

(a and b have no relation, but were arbitrarily designated at the beginning)

1	a	8	2d	(10)	8	a	6	2	8	15	a	7	d	(7)	22	a	7	1d	(8)
	b	7	4	11		b	9	d	(9)		b	7	2	9		b	7	d	(7)
2	a	6	1d	(7)	9	a	8	d	(8)	16	a	6	1	7	23	a	4	0	4
	b	8	1	9		b	7	1	8		b	9	2	11		b	7	2	9
3	a	8	1d	(9)	10	a	9	1	10	17	a	8	3	11	24	a	8	3	11
	b	10	4	14		b	7	1	8		b	6	1d	(7)		b	9	2	11
4	a	11	4	15	11	a	2d		(9)	18	a	8	2d	(10)	25	a	6	3	9
	b	19	2	12		b	5	2	7		b	9	1d	(10)		b	9	d	(9)
5	a	7	2	9	12	a	10	3	13	19	a	10	4	14	26	a	d		(10)
	b	7	2	9		b	8	2d	(10)		b	d		(6)		b	7	3	10
6	a	6	2d	(8)	13	a	9	2	11	20	a	9	1d	(10)	27	a	9	1d	(10)
	b	9	3	12		b	9	1	10		b	8	2	10		b	7	d	(7)
7	a	9	3	12	14	a	7	2	9	21	a	d		(9)	28	a	9	2d	(11)
	b	7	1d	(8)		b	6	d	(6)		b	6	1d	(7)		b	8		(10)
															29	a	6	d	(6)
																b	7	d	(7)

TABLE 32

Experiment 12. Paramecium aurelia. Number of individuals present on certain days of the experiment, with the reductions made. Such entries as '14-4' signify that 14 were present on the day in question, and that a sufficient number were removed to leave but 4 for future multiplication. No individuals were removed except in the numbers and on the dates shown. Each line started with a single individual, August 11.

Set K. Progeny of the conjugants of June 3. Cultivated in watch glasses since May 15.

LINE	AUGUST						SEPTEMBER						TOTAL FISSIONS
	14	15	20	25	27	29	1	2	3	5	6	7	
1	6-2	8-2	16-4	8-4	8-4	12-4	16-4	6-4	13-4	14-4	7	13	21
2	8-2	6-2	16-4	20-4	8-4	16-4	21-4	6-4	8-4	30-4	8	19	23
3	8-2	8-2	16-4	13-4	8-4	16-4	32-4	3	discontinued				(15)
4	2	12-2	21-4	12-4	8-4	lost							
5	9-2	8-2	12-4	10-4	11-4	13-4	19-4	4	discontinued				(16)
6	8-2	8-2	12-4	11-4	7-4	18-4	29-4	6-4	8-4	19-4	8	14	22
7	12-2	4-2	8-4	15-4	6-4	10-4	12-4	8	discontinued				(14)
8	7-2	4-2	5-4	14-4	11-4	12-4	13-4	6-4	4	8-4	6	8	15
9	5-2	7-2	32-4	12-4	8-4	16-4	14-4	6	discontinued				(16)
10	14-2	4-2	13-4	15-4	16-4	16-4	20-4	7-4	8-4	16-4	7	12	22

Set D 1. Conjugants of August 12, from the same culture as the non-conjugants of set D 2. Cultivated on slides from March 4 till June 7; from that time in watch glasses. (1 a and 1 b constitute the two members of pair 1, etc.)

1 a	8-2	2	3-2	23-4	6	8	8	5	5	5	2	d	8
	-2	10	22	45-10	9	12	discontinued						(8)
	-2	8-2	8	3	d								7
	-2	8-2	10	d									8
1 b	d	2	d										2
2 a													0
2 b	8-2	2	7	77-10	19	67	discontinued						(13)
	-2	2	11	8-4	2	d							6
	-2	2	10	14-4	5	13	26	22-4	6	8	8	8	11
								-4	6	10	10	10	12
3 a								-4	6	11	10	12	12
								-4	5	9	10	11	12
	1	d											0
	8-2	4-2	3	d									5
3 b	-2	2	d										3
	-2	8-2	6	9-4	6	7	26	26-4	4	14	13	13	12
								-4	7	13	11	14	12
								-4	4	12	13	13	12
								-4	4	9	11	11	12
								-4	4	14	15	15	12

TABLE 32 (CONTINUED)

Set D 2. Non-conjugants of August 12, from same culture as the conjugants of set D 1. Cultivated on slides from March 4, till June 7; from that time in watch glasses.

LINE	AUGUST						SEPTEMBER							TOTAL FISSIONS
	14	16	20	25	27	29	1	2	3	5	6	7		
1	8-2	6-2	4	1	1	d							6	
2	8-2	3-2	4	7-4	4	4	3	1	d				6	
3	d												0	
4	8-2	2	5	13-4	4	d							6	
	-2	4-2	4	9-4	6	1	1	d					7	
5	4-2	4-2	5	d									6	
	4-2	8-2	d										6	
6	d												0	
7	6-2	4-2	4	d									5	
8	4-2	8-2	4	d									5	
9	7-2	4-2	4	d									5	
10	2	8-2	8	2	4	8	7	6	4	2	d	d	7	
									2	2	1*		7	

TABLE 33

Experiment 13 b. *Paramecium aurelia*. Comparative number of fissions in the selected conjugant and non-conjugant lines, for five periods, between December 8 and February 27. (d = died out.)

LINE	DESCENDANTS OF CONJUGANTS						LINE	DESCENDANTS OF NON-CONJUGANTS					
	December 8-26	January 2-14	January 15-29	January 30-February 12	February 13-27	First 3 periods		December 8-26	January 2-14	January 15-29	January 30-February 12	February 13-27	First 3 periods
1	21	11	14	9	13	40	1	20	12	18	14	12	50
2		19	12	13	9	3	2	21	11	14	12	9	46
3		20	9	14	12	7	3	15	12	15	12	12	42
4		18	10	13	14	9	4	17	11	14	12	d	42
5		14	11	15	13	10	5	17	8	16	12	11	41
6		13	11	13	15	d	6	15	11	15	13	11	41
7		12	10	13	15	11	7	19	10	11	9	9	40
8		14	8	13	d	5	8	16	11	13	11	6	40
9		14	8	11	d	33	9	15	9	15	13	10	39
10		11	9	11	10	8	10	16	11	12	d		39
11		14	7	9	7	2	11	12	10	14	12	8	36
12		11	10	8	d	29	12	13	9	13	12	12	35
13		5	10	13	12	3	13	15	9	4	d		28
14		10	7	4	5	d	14	10	8	6	5	3	24
15			4	8	10	2							
16		9	7	5	7	0							
17		6	d										
18		d											
19		d											
20		d											
21		d											

TABLE 34

Experiment 15. Pure strain E; conjugants. Record of number of fissions by two-day periods, for each of the 88 lines descended from 28 conjugating pairs, throughout the twenty-four days of the experiment. For full explanation, see 'Explanation of tables 34 and 35,' page 348.

LINE	AUGUST				SEPTEMBER								TOTAL 24 DAYS
	27	29	31	2	4	6	8	10	12	14	16	18	
1 a x.....	0	2	2	2	3	4	4	4	3	3	3	3	33
y.....	0	2	1	2	4	1	2	4	3	3	3	2	27
b x.....	0	3	1	2	4	3	3	4	3	3	3	2	31
y.....	0	3	1	0	3	2	2	3	3	2	3	1	23
2 a x.....	2	1	2	1	3	3	3	3	3	3	3	2	29
y.....	2	2	2	1	4	2	4	3	3	3	3		31
b x.....	3	3	1	1	3	3	4	3	3	3		3	
y.....	3	2	1	2	3	3	2	3	3	3	2	3	30
3 a x.....	2	3	1	3	1	3	2	4	3	3	3	3	34
y.....	2	3	1	2	4	3	4	3	2	3	4	3	34
b x.....	2	2	2	2	4	3	3	4	2	3	3	2	32
y.....	2	3	0	2	4	3	4	4	2	3	3	3	33
4 a x.....	3	2	2	2	1	1	0	1	0	1	0	0	13
y.....	3	2	2	2	2	0	0	1	0	0	0	0	12
b x.....	2	2	2	2	2	1	0	1	0	0	0	0	12
y.....	2	2	2	2	2	1	1	1	0	0	0	0	13
5 a x.....	3	3	2	1	0	1	0	1	0	0	0	0	11
y.....	3	2	2	1	0	0		1	0	0	0	0	
b x.....	0	2	2	2	2	1	0	1	0	0	0	0	10
y.....	1	2	2		2	0	1	1	0	0	1	0	
6 a x.....	0	2	1	1	1	1	1	2	2	2	1	2	16
y.....	0	2	1	1	1	1	1	2	2	2	1	2	1
b x.....	0	1	2	1	2	2	2	2	2		1	2	
y.....	0	1	2	2	2	1	2	2	1	2	2	0	17
7 a x.....	0		1	2	3	4	3	3	3	3	3	3	(30)
y.....	0	2	2	2	4	4	3	3	3	3	3	3	32
b x.....	0		2	2	4	3	3	4	3	3	3	3	(33)
y.....	1	2	1	2	4	3	4	3	3	3	3	3	32
8 a x.....		2	0		3	3	3	3	3	3	3	3	
y.....	1	2	1	2	3	3	3	4	2	2	3	3	29
b x.....	0	2	2	1	2	3	3	3	3	2	3	3	27
y.....			2		3	3	2	4	2	3	3	3	
9 a x.....	1	2	2	3	4	4	3	3	3	3	3	4	35
y.....	1	2	2	2	3	4	4	4	3	3	3	3	34
b x.....	2	2	2	1	3	3	4	3	3	3	3	3	32
y.....	1	2	0	0		4		4	3	3	3	4	
10 a x.....	2	2	2	2	3	2	3	3	3	3	3	1	29
y.....	1	3	1		4	3	3	4	2	3	3	2	

TABLE 34 (CONTINUED)

LINE	AUGUST				SEPTEMBER								TOTAL 24 DAYS
	27	29	31	2	4	6	8	10	12	14	16	18	
10 b x.....	2	2	2	1	3	3	4	3	3	2	3	3	31
y.....	2	3	0	2	3	2	3	3	3	3	3	3	30
11 a x.....	2	2	2	2	4	3	4	3	2	3	3	2	32
y.....	2	3	0	3		3	3	3	1	1	3	2	
b x.....	2	2	1	2	3	3	4	3	3	3	3	2	31
y.....	2	2	1	2	3	2	4	2	3	2	2	3	28
12 a x.....	2	2	2	2	4	3	3	3	3	2	4	3	33
y.....	2	2	2	2	3	3	3	3	3	3	3	3	32
b x.....	3	1	1	2	3	2	3	2	3	3	3	3	29
y.....	3	2	1	0	2	3	1	3	3	2	3	2	25
13 a x.....	2	3	1	2	3	2	3	3	3	2	3	2	29
y.....	2	2	1		3	2	3	3	3	2	3	2	
b x.....	2	2	1	1	3	2	2	3	3	2	3	2	26
y.....	2	2	0		2	1	3	3	2			2	3
14 a x.....	2	2	2	2	3	3	3	3	3	3	2	4	32
y.....	2	2	1	2	3	2	3	4	3	2	3	2	29
b x.....	2	2	2	2	3	3	3	3	3	3	2	3	31
y.....	2	2	1	2	3	3	3	3	3	3	2	3	30
15 a x.....	2	2	2	2	1	2	0	1	0	1	0	0	13
y.....	0		2	1	2	0	0	1	0	0	0	0	(10)
b x.....	2	3	2	0	1	1	1	1	0	0	0	0	11
y.....	2	3	2	2	2	1	1	1	0	1	0	0	15
16 a x.....	2	3	1	2	3	3	4	4	3	2	3	3	33
y.....	2	3	1	2	4	1	4	4	3	0		3	
b x.....	2	2	2	1	5	3	3	4	2	3	3	3	33
y.....	2	2	1	2	4	3	3	3	3	3	0	1	27
17 a x.....	0	0	2	3	3	2	4	3	3	3	3	2	28
y.....	0	0	2	3	3	4	3	3	3	3	3	3	30
18 a x.....	1	2	1	2	4	3	3	4	3	3	3	3	32
y.....	0	2	2	1	3	3	2	4	2	2	3	3	27
19 a x.....	2	2	2	1	3	3	2	4	3	2	3	3	30
y.....	2	2	2	0	2	1	3	3	3	2	3	3	26
20 a x.....	1	2	1	2	3		3	3	3	2	3	3	
y.....	0	2	1	2	3	3	1	3	3	2	3	3	26
21 a x.....	2	2	1	1		1	2	3	3	3	3	3	
y.....	2	3	1	2	4	2	4	4	3	3	3	3	32
22 a x.....	2	1	2	1	4		3	3	1	3	3	3	
y.....	2	2	2	2	2	3	3	3	1	3	3	2	28
23 a x.....	2	3	2	2	4	3	3	4	3	2	3	3	34
y.....	2	3	2	2	4		3	3	3	3	3	4	
24 a x.....	2	2	1	1	4	2	3	3	3			3	
y.....	2	2	2	1	4	1	2	3	2	2	3	3	27
25 a x.....	2	2	2	2	4	3		4	3		4	2	
y.....	2	2	2	3	4	2	4	4	3	2	3	3	34

TABLE 34 (CONTINUED)

LINE	AUGUST				SEPTEMBER								TOTAL 21 DAYS
	27	29	31	2	4	6	8	10	12	14	16	18	
26 a x.....	2	2	2	3	2	1	1	1	1	0	1	0	16
y.....	2	3	1	2	0	1	1	0	1	0	0	0	11
27 a x.....	1	3	1	1	2	2	2	2	2	2	3	1	22
y.....	1	3	0		1		1	2	2	2	2	2	
28 a x.....	2	3	0	0	2	2	2	3	3	3	2	3	25
y.....	2	3	0	2	4	2	2	3	3	2	3	3	29

TABLE 35

Experiment 15. Pure strain E; non-conjugants. Record of number of fissions by two-day periods, for each of the 174 lines descended from 65 split pairs, throughout the 21 days of the experiment. For full explanation, see 'Explanation of tables 34 and 35' page 346.

LINE	AUGUST				SEPTEMBER								TOTAL 21 DAYS
	28	30	1	3	6	8	10	12	14	16			
1 a x.....	3	1	1	2	4	4	2	3	2	3			25
y.....	3	0	2	2	4	4	2	3	2	3			25
b x.....	1	1	2	3	4	3	2	3	1	3			23
y.....	1	1		3	4	2	3	2	2	3			
2 a x.....	3	1	2	2	4	3	2	2	3	2			24
y.....	3	1	2	3	4	3	2	3	2	2			25
b x.....	2	1	1	2	3	4	2	3	2	3			23
y.....	2	1	2	2	4	3	3	3	2	3			25
3 a x.....	3	0	2	2	3	1	1	2	2	2			18
y.....	3	2	2	2	1	2	2	2	2	3			24
b x.....	2	1	2	3	4	3	2	2	2	3			24
y.....	1	1	1	2	4	3	3	2	2	3			22
4 a x.....	2	2	2	2	4	2	3	2	2	3			24
y.....	3	2	1	2	3	2	2	2	2	3			22
b x.....	2	3	2	2	3	3	3	2	3	2			25
y.....	2	2		2	4	3	2	3	0				
5 a x.....	3	2	1	2	3	3	2	3	2	3			23
y.....	3	2	2	2	1	3	3	2	3	2			23
b x.....	2	2	2	3	4	2	2	2	2	3			24
y.....	2	1	2	3	4	4	2	2	2	3			25
6 a x.....	1	1		3	3	3	2	3	2	1			
y.....	2	2	1	3	2	3	2	2	2	3			22
b x.....	1	3	2	2	4	3	2	3	2	3			25
y.....	1	2	3	2	4	4	3	2	3	3			27
7 a x.....	2	1	2	2	3	3	3	2	2	2			22
y.....	0	2	1		3	2	2	2	2	2			

TABLE 35 (CONTINUED)

LINE	AUGUST		SEPTEMBER										TOTAL 21 DAYS
	28	30	1	3	6	8	10	12	14	16			
7 b x.....	3	1	2	3	3	4	3	2	3	2			26
y.....	3	2	2		3	4	3	3	2	3			
8 a x.....	2	2	0	1	3	3	3	2	2	3			21
y.....	2	2		3	3	2	3	2	2	3			
b x.....	3	1	2	2	4	3	2	3	2	3			25
y.....	3	0	1	2	4	3	3	3	2	3			24
9 a x.....	1		2	2	3	4	3	2	3	2			
y.....	2	2	2	2	4	3	2	3	2	2			24
b x.....	2	2	2	3	3	2	3	2	3	3			25
y.....	2	2	2	2	4	3	3	2	3	2			25
10 a x.....	2	1	1	2	3	4	2	3	2	2			22
y.....	2	2	2	2	3	3	3	2	3	2			24
b x.....	2	2	3	2	5	2	3	2	2	3			26
y.....	3	2	2	2	4	3	3	2	3	2			26
11 a x.....	3	2	0	2	3	3	3	2	2	3			23
y.....	2	2	1	2	3	3	2	2	2	3			22
b x.....	2	2	2	3	3	3	3	1	2	3			24
y.....	2	2	1	3	3	4	2	3	2	3			25
12 a x.....	0	3	2	2	4	3	3	3	2	3			25
y.....	2	2	1	2	3	3	3	3	2	2			23
b x.....	2	1	2	3	4	2	3	3	2	2			24
y.....	2	0	2	3	3	3	2	2	2	3			22
13 a x.....	2	2	2	2	3	2	3	2	2	3			23
y.....	2	1	2	2	3	3	3	2	1	1			20
b x.....	2	2	0	1	4	3	2	3	2	2			21
y.....	2	2	2	2	4	3	2	3	2	2			24
14 a x.....	2	3	2	3	3	3	2	3	2	3			26
y.....	3		1	2	3	2	2	3	2	3			
b x.....	3	2		1	3	2	3	2	2	3			
y.....	3	1	3	2	3	3	2	3	1	3			24
15 a x.....	2	2	2	2	3	3	3	2	2	3			24
y.....	2	3	2	1	4	2	2	2	2	3			24
b x.....	0	2	0	2	4	4	3	3	2	3			23
y.....	0	0		3	4	3	3	2	2	3			
16 a x.....	3	2	1	2	4	3	3	3	2	3			26
y.....	2	2	2	3	4	3	2	3	2	2			24
b x.....	0		2	2	4	3	3	2	3	2			
y.....	0	3	1	2	4	3	3	2	2	3			23
17 a x.....	2	3	2	2	4	3	3	2	2	2			25
y.....	3	1	1	2	4	3	2	2	2	3			23
b x.....	0	3	2	2	4	3	3	3	2	2			24
y.....	0		2	2	4	3	2	2	2	2			
18 a x.....	3	2	2	3	3	3	3	2	2	3			26
y.....	2	3	1	3	4		3	2	2	3			

TABLE 35 (CONTINUED)

LINE	AUGUST		SEPTEMBER								TOTAL 21 DAYS
	28	30	1	3	6	8	10	12	14	16	
18 b x.....	0	2	2	2	4	3	3	2	3	3	24
y.....	0	1	2	4	3	3	3	2	3	3	
19 a x.....	2	2	2	2	4	3	2	3	2	3	25
y.....	2	2	1	3	3	2	3	2	3	2	23
b x.....	1	2	1	3	4	3	3	3	2	3	25
y.....	1	2	3	4	1	3	2	3	3	3	
20 a x.....	2	3	2	2	3	3	3	3	2	2	25
y.....	3	1	3	2	3	3	3	2	2	3	
b x.....	0	1	2	4	3	4	2	2	3	3	
y.....	1	1	1	2	4	3	3	2	2	3	22
21 a x.....	2	2	3	2	4	2	3	3	2	3	26
y.....	2	2	2	2	4	3	3	2	3	3	26
b x.....	0	2	1	3	4	3	2	3	2	3	23
y.....	0	1	2	2	4	3	3	1	2	3	21
22 a x.....	2	3	3	2	4	3	3	3	2	3	28
y.....	2	3	2	3	3	3	2	3	2	3	26
b x.....	0	2	2	3	4	4	2	3	3	3	26
y.....	0	1	3	4	3	2	3	2	3	3	
23 a x.....	3	2	2	2	4	1	2	2	2	3	23
y.....	2	1	3	2	3	2	2	2	3	3	23
24 a x.....	1	2	1	2	3	4	2	3	2	1	21
y.....	3	1	1	2	1	3	2	3	2	3	24
25 a x.....	2	2	2	2	3	4	2	3	2	3	25
y.....	2	3	1	3	4	2	2	3	2	3	25
26 a x.....	2	2	1	1	3	3	2	3	2	3	22
y.....	3	0	2	3	3	3	2	2	3	3	
27 a x.....	3	2	2	2	4	2	3	3	1	3	25
y.....	3	2	1	2	3	3	3	2	2	2	23
28 a x.....	3	1	2	2	3	3	2	3	2	3	24
y.....	3	2	2	4	3	2	3	2	3	3	
29 a x.....	3	2	2	2	3	3	3	3	2	2	24
y.....	2	2	2	3	3	3	2	2	2	3	24
30 a x.....	3	1	2	2	3	3	3	2	3	2	24
y.....	2	2	2	2	4	3	3	3	2	3	26
31 a x.....	3	2	2	2	4	2	3	2	2	1	23
y.....	4	0	2	2	4	3	2	2	2	3	24
32 a x.....	2	1	1	2	4	2	2	2	2	2	21
y.....	2	2	2	2	4	3	3	2	2	3	25
33 a x.....	2	2	2	2	4	3	2	2	2	3	24
y.....	3	2	2	2	4	3	2	3	2	3	26
34 a x.....	3	0	2	1	3	3	2	2	2	2	20
y.....	2	2	1	2	4	3	3	2	2	3	24
35 a x.....	3	2	2	2	4	3	2	3	2	3	26
y.....	3	0	3	3	3	3	2	2	3	3	23

TABLE 35 (CONTINUED)

LINE	AUGUST				SEPTEMBER							TOTAL 21 DAYS
	28	30	1	3	6	8	10	12	14	16		
36 a x.....	3	2	2	2	4	2	2	2	2	2	23	
y.....	3		1	3	3	3	2	3	2	3		
37 a x.....	2	2	1	3	3	4	2	3	2	3	25	
y.....	2	2	2	3	4	2	3	2	3	3	26	
38 a x.....	2	1	2	3	3	3	2	3	2	3	24	
y.....	2	1	1	3	4		2	3	2	2		
39 a x.....	1	3	2	1	3	3	3	2	2	3	23	
y.....	2	2	2	2	4	3	2	3	2	2	24	
40 a x.....	2	2	2	3	4	3	3	2	2	3	26	
y.....	2	2	3	2	4	3	3	3	2	3	27	
41 a x.....	2	2		2	5		2	3	2	3		
y.....	2	2	2	2	5	3	3	2	2	2	25	
42 a x.....	1	2	1	2	4	3	2	3	2	2	22	
y.....	1	2	3	2	4	3	3	3	2	2	24	
43 a x.....	2	2	2	2	4	3	3	2	2	2	24	
y.....	3	2	2	2	4	3	3	3	1	2	25	
44 a x.....	2	3	2	3	4	3	3	2	2	3	27	
y.....	2		1	3	4	2	3	2	2	2		
45 a x.....	1	1	1	2	4	2	2	2	2	2	19	
y.....	2	2	1	3	4	3	2	2	2	2	23	
46 a x.....	2	2		2	3	3	3	2	2	2		
y.....	2	2	2	2	4	2	2	3	2	2	23	
47 a x.....	2	1	2	2	4	3	2	2	2	3	23	
y.....	3	2	2	2	4	3	3	2	2	3	26	
48 a x.....	2	2	2	2	4	3	3	2	2	3	25	
y.....	2	2	2	2	4	3	2	2	2	2	23	
49 a x.....	3	0	2	3	4	3	2	3	2	2	24	
y.....	3	2	2	2	4	2	2	2	2	3	24	
50 a x.....	2	2	2	3	4	3	3	3	2	2	25	
y.....	2	1	0	3	4	3	3	1	2	3	22	
51 a x.....	3	2	3	2	4	3	3	2	2	3	27	
y.....	3	1	3	2	3	3	3	2	2	3	25	
52 a x.....	3	2	1	2	3	2	3	2	3	3	24	
y.....	2	3	1	2	4	3	3	2	2	3	25	
53 a x.....	3	2	2	2	3	3	3	2	3	3	26	
y.....	3	1	3	3	3	3	3	2	2	3	26	
54 a x.....	1	2	2	2	3	3	3	2	2	3	23	
y.....	2	2	2	2	3	1	2	2	2	3	21	
55 a x.....	2	2	2	2	3	2	3	2	2	3	23	
y.....	2	3	2	2	4	3	3	2	2	3	26	
56 a x.....	2	1	2		3	3	3	2	2	2		
y.....	2	2	2	3	2	2	3	2	2	3	23	
57 a x.....	2	1	2	3	3	2	3	2	2	2	22	
y.....	3	2	2	3	3	3	3	2	2	3	26	

EFFECT OF CONJUGATION

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TABLE 35 (CONTINUED)

LINE	AUGUST		SEPTEMBER								TOTAL 21 DAYS
	28	30	1	3	6	8	10	12	14	16	
58 a x.....	1	3	2	3	4	2	3	2	2	3	25
y.....	3	2	2	3	4	3	3	2	2		
59 a x.....	2	1	2	2	3	3	3	2	2	3	23
y.....	2	2	3	2	3	3	3	2	2	2	24
60 a x.....	2	2	2	3	4	3	2	3	2	3	26
y.....	3	1	2	3	3	3	2	2	2	3	24
61 a x.....	2	2	2	2	4	4	3	2	2	3	26
y.....	2	2	2	2	3	4	3	2	2	3	25
62 a x.....	2	2	2	2	3	2	3	2	2	3	23
y.....	2	2	1	3	3	3	3	2	3	3	25
63 a x.....	1	2	1	3	3	2	3	2	2	3	22
y.....	2	2	2	3	3	4	3	2	3	2	26
64 a x.....	2	3	2	2	3	4	2	3	2	3	26
y.....	2	1	2	2	4	3	3	2	3	3	25
65 a x.....	2	2	2	3	3	3	3	2	2	2	24
y.....	2	2	2	2	3	3	3	2	2	3	24

BIPARENTAL INHERITANCE AND THE QUESTION OF SEXUALITY IN PARAMECIUM¹

H. S. JENNINGS AND K. S. LASHLEY

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TWO FIGURES

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¹ The results in the present paper are based to a certain extent on an analysis of data presented in the tables of the preceding paper, on The Effect of Conjugation in Paramecium (this Journal, vol. 14, 1913, page 279). To facilitate reference to the tables in that paper and to avoid confusion, the tables in the present contribution are numbered consecutively with those of the former paper, beginning thus with table 36. All references to tables numbered below 36 are therefore to those in the preceding paper.

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PART I

H. S. JENNINGS

INTRODUCTION

When the individuals of *Paramecium* that have paired are kept separate and allowed to reproduce under favorable conditions, as a rule a considerable number of the lines of progeny produced by them either die out, or reproduce very slowly; while the others live and reproduce freely. This has been studied by Calkins ('02) and by Cull ('07) and is held by them to indicate that there is at least an incipient sexuality in *Paramecium*. Of the two members, *a* and *b*, of a pair, one is held to reproduce freely, thus corresponding to the female, while the other, reproducing little or not at all after conjugation, represents the male. Since the life of individuals that do not reproduce is short, the 'males' will in many cases die in a brief period after conjugation.

METHOD OF ANALYSIS: FORMULAE

Now, of course the mere fact, in itself, that a considerable number of the lines of progeny are weak or die out after conjugation does not show that there is a tendency to sexual differentiation in the members of pairs. There might be causes of weakness or death that are quite independent of such sexual differentiation. How can we determine whether the observed cases of weakness and death do indicate a sexual differentiation?

For answering this question the following considerations apply: If the causes of death are quite independent of sexual differentiation between the two members of the pairs, then sometimes both members of a given pair will die or be weak, and it should be pos-

sible to determine how frequently, on the average, this will occur. This would enable us to determine further in how large a proportion of the cases both members of pairs should be represented among the survivors, in this case where the distribution of deaths has no relation to the pairing.

On the other hand, if the weakness and death are due to the fact that one member (the 'male') of a pair does not reproduce well, then it would be unusual for both members of a pair to show weakness or death; from this cause acting alone it would never occur. There would probably, of course, be acting also other causes of weakness or death, that were independent of the pairing, so that we should sometimes find that both members of a pair die or are weak, owing to chance causes, but *the number of cases where both members die would be smaller, in proportion to the total number of deaths, than if the distribution of deaths were purely random so far as the pairing goes.* For one of the chief causes for the deaths would affect only one member of each pair. Thus among the survivors there would be a greater proportion of cases where only one member of the pair exists than would be the case if sexual differentiation played no part in the matter.

This gives us a method of testing the matter. If, owing to sexual differentiation, one member of the pair is more liable to weakness or death than the other, then the number of cases where both members of the pair survive will be found less than would be probable if the distribution of deaths were due to causes that were independent of the pairing.

To take a concrete case, Miss Cull ('07) found that out of the progeny of 93 pairs (186 lines), there remained at the end of a month 103 lines, and among these 103 lines were representatives of both members in 38 pairs. Is this number of entire pairs still living less than would be expected if the distribution of deaths had no relation to the pairing?

The same question may be dealt with by taking up the cases that die, in place of those that survive. Thus, in the case cited above, out of the progeny of the two members of 93 pairs (186 lines), 83 lines had died out, including 28 cases where both members of the pairs had died. Is this number of pairs dead less than

would be expected if the distribution of the 83 deaths had no relation to the method of pairing?

It is important to realize that these two ways of putting the question are identical. The probability that 38 complete pairs should survive is in this case the same as the probability that 28 complete pairs should die, and this is true in general. Therefore we need deal explicitly with only one of these questions; the answer we obtain will hold for both. This will be demonstrated later.

Miss Cull ('07) appears to leave out of consideration all pairs in which both members die, dealing only with those which survive. At the end of a month, since 28 pairs out of the 93 had died out, it follows that there were left representatives of but 65 pairs, and it is only these 65 that she considers in summing up the evidence in favor of sexual differentiation:

It may be broadly stated that of the sixty-five pairs which I have observed one conjugant either died or left a weak strain in which the descendants were half as numerous and much less vigorous than those of the stronger ex-conjugant. . . . Here we have indications that one gamete gives up its vitality to and loses its identity in the egg where its presence forms a stimulus to development analogous to the *rajeunissement* and greater activity in cell division which follows conjugation. There is little reason to doubt that a physiological and perhaps a physical difference exists between the two unicellular organisms which unite in conjugation and a difference of the same nature as that expressed morphologically in the case of *Adelea ovata*, where the male gamete does not fuse with the female, but dies after delivering one of its four pronuclei (pp. 88, 89).

Now, even if we deal only with the survivors, as in our first way of putting the question, we come to the same result as when we deal only with those that die (as set forth above, and as will be demonstrated later). But in any case, the results given in my paper on the effects of conjugation ('13) show that there is no justification for omitting the cases in which both members of the pair die. Miss Cull did this apparently on the ground that these were instances where conjugation was unsuccessful in producing rejuvenescence, so that the animals died as they would have done if there had been no conjugation. She says of the death of these 28 pairs: "These facts confirm Calkins' observation

that conjugation is by no means always successful in producing rejuvenescence" (p. 87). But I have shown in the paper ('13) which precedes this one that there is no ground for supposing that these would have died if conjugation had not occurred; in our Experiment 1, for example, none of those that were prevented from conjugating died, the deaths being limited to those that did conjugate; and the other experiments give evidence in the same direction. This takes away all ground (if there ever was such ground) for trying to exclude the cases in which both members of the pairs died. What we must inquire, so far as deaths go, is, whether the number of complete pairs that survive (or, if we prefer, the number of complete pairs that die) is less than would be expected if the deaths were due to causes that had no relation to the pairing.

To answer this question, we must know, first, how many complete pairs would probably have occurred among the survivors (or the non-survivors), if the deaths had taken place at random. In Miss Cull's case, cited above, we must ask: How many complete pairs would have survived among the 103 lines (out of 186), if the deaths had occurred at random? Would the number have been greater or less than 38 (the actual number)? Or: How many complete pairs would have died among the 83 deaths (out of 186), if the deaths had no relation to the pairing?

To deal with this and similar cases, we are compelled to take up this general problem: Suppose that we have a given number of pairs, from which a given number of individuals are drawn at random; what is the most probable number of cases where both members of pairs will be drawn? We may realize such a case concretely by throwing a lot of serially numbered tickets into a hat, there being two tickets bearing each number (these constituting a pair), then drawing out a certain number of the tickets. What we wish is a formula for determining how many entire pairs (both members) we shall probably get for any given number of tickets taken from the hat. This will at the same time determine how many pairs will be left. The question may be put algebraically thus:

Given m numbers, forming pairs; from these n numbers are drawn. What is the most probable number of entire pairs that will be drawn? And what is, consequently, the most probable number of entire pairs that will be left?

This problem I did not find explicitly taken up in any of the books on probabilities which I consulted. It may be attacked directly in the following way:

Suppose that the total number m is 20 (forming thus 10 pairs), so that we have in the hat the series 1 to 10, twice repeated. Now, if we draw out one number, obviously no pair will be obtained. There are then 19 numbers left, and if we draw out one more, there is one among the 19 that will, with the first one drawn, make one pair. Thus the chance for getting one pair when two members are drawn is in this special case $1/19$, and in general, it is $\frac{1}{m-1}$. That is, if we repeat the process of drawing 2 from 20

a great number of times, we shall get a pair in $1/19$ (or $\frac{1}{m-1}$) of all cases, while we shall get no pair at all in the remainder, or $18/19$ ($= \frac{m-2}{m-1}$), of all the cases.

Now, consider the case where one more number is drawn, making 3. Before the third one is drawn, there remain 18 (or $m-2$). Now, as we have already seen, a pair will have been obtained with the first two drawn in $1/19$ ($= \frac{1}{m-1}$) of all cases; in these cases no additional pair can be obtained when the third is drawn. But in the cases where the first two drawn did not form a pair (that is, in $18/19$, or $\frac{m-2}{m-1}$, of all cases), there are two numbers out of the 18 (or $m-2$) remaining that, with the two already drawn, will form pairs. Thus there are now two chances out of 18 for getting a pair when the third number is drawn. But this is true only for $18/19$ (or $\frac{m-2}{m-1}$) of all cases. So the total chance from this source for drawing a pair is $2/18$ of $\frac{18}{19}$ (or $\frac{2}{m-2}$ of $\frac{m-2}{m-1}$), which

equals $2/19$, (or $\frac{2}{m-1}$). We had found from the previous source a chance of $1/19$ for a single pair, so the total chance for one pair when 3 are drawn is $1/19 + 2/19$, or $3/19$ (in general, $\frac{1}{m-1} + \frac{2}{m-1} = \frac{3}{m-1}$). The remainder of the chances ($16/19$, or $\frac{m-4}{m-1}$) are for 0 pairs when 3 are drawn. Thus, when we draw 3 from a given number m , and repeat the drawing many times, the average number of pairs obtained will be $\frac{3}{m-1}$.

By a continuation of this line of reasoning, which becomes somewhat complicated as the number drawn becomes larger, we discover that the average number of pairs that will be obtained when n units are drawn is

$$\frac{1+2+3+4 \dots \text{to } (n-1)}{m-1} = \frac{\frac{1}{2}n(n-1)}{m-1}$$

Thus, when 9 are drawn from 20, the average number of pairs to be obtained, if the drawing is repeated an indefinitely great number of times, is

$$\frac{\frac{1}{2}(9 \times 8)}{19}, \text{ or } \frac{36}{19} = 1 \frac{17}{19}$$

This average, like any other average, would be obtained in a concrete case, by multiplying each number of pairs by the number of times it is drawn, and dividing by the number of drawings. Thus, in the case given above, if there are a great number of drawings of 9 from 20, the various number of pairs will be obtained in the following proportions:

0 pairs in 384 drawings	(= 0 pairs)
1 pairs in 3,456 drawings	(= 3,456 pairs)
2 pairs in 6,048 drawings	(= 12,096 pairs)
3 pairs in 2,520 drawings	(= 7,560 pairs)
4 pairs in 189 drawings	(= 756 pairs)
12,597 drawings	23,868 pairs

Thus a total of 23,868 pairs will have been obtained in 12,597 drawings, giving an average of $1 \frac{17}{19}$ (or 1.8947) pairs for one drawing. In an actual case of 100 drawings of 9 from 20, the average was 1.91 pairs.

This gives us our first formula. If we let k be the average number of pairs, our formula for determining it is

$$k = \frac{\frac{1}{2} n (n-1)}{m-1} \quad (1)$$

As a rule this average number of pairs gives also the most probable number of pairs that will be drawn—this being the integral number nearest the average. In the above case, for example, the most probable number of pairs when 9 are drawn from 20 is 2. In Miss Cull's case, already cited, where 83 died out of 186, the average number of pairs included would be $\frac{\frac{1}{2}(83 \times 82)}{185}$, or 18.39, so that the most probable number is 18 (in place of 28, the number actually found). (Five actual drawings of 83 tickets from 186 gave respectively 16, 18, 18, 18, 20 pairs, the most frequent being 18, as our formula demands.) For most practical purposes formula (1) is quite adequate for determining the most probable number of pairs.

But it happens in rare cases that the integer nearest the average is not the most probable number of pairs. It is apparently always within 1 of the probable number, and this is usually a sufficiently close approximation, since the probability will be nearly the same for the two numbers that differ only by unity. To take an example, when we draw 5 from 20, the average number of pairs to be obtained from repeated drawings is $\frac{\frac{1}{2}(5 \times 4)}{19} = \frac{10}{19}$. Now, the nearest integer to $\frac{10}{19}$ is 1, yet complete analysis shows that 0 is slightly more probable than 1 (the relative probabilities of 0, 1 and 2 pairs are in this case as 168, 140 and 15).

For a formula which for *even numbers drawn* gives correctly the most probable number of pairs I am indebted to Dr. A. B. Coble, of the Mathematical Department of The Johns Hopkins University. This formula is (if k represents the most probable number of pairs):

$$k = \frac{(n+1) \left(\frac{n}{2} + 1 \right)}{m+3} \quad (2)$$

In this case the nearest integer below the result gives the most probable number of pairs (if the result is itself integral, then this and the integer below it are equally probable). Thus, if we draw 10 from 20, the most probable number of pairs is given by

$$k = \frac{11 \times 6}{23} = 2 \frac{20}{23}$$

so that the most probable number of pairs is 2. But this formula is not available when odd numbers are drawn. We shall see later an indirect method of determining with absolute certainty the most probable number of pairs when an odd number of units is drawn.

But it is often needful to know what is the relative probability of a given number of pairs being drawn, even though this may not be the most probable number. For example, in Miss Cull's case, cited above, we found that the most probable number of entire pairs that will be included when 83 out of 186 die is 18, while the actual number of pairs included is 28. What is the probability that we should get 28, if the distribution of deaths has no relation to the pairing?

For a formula to determine the probability of any given number of pairs when a given number of units is drawn, I am again indebted to Dr. A. B. Coble, to whom I wish to express my thanks. If x represents the probability of any given number of pairs k , then Coble's formula is as follows:

$$x = \frac{n! m-n! l! 2^{n-2k}}{m! k! n-2k! l-n+k!} \quad (3)$$

where

- m = the total number of individuals
- l = the total number of pairs (so that $l = \frac{1}{2} m$)
- n = the number of individuals drawn
- k = the number of pairs whose probability we desire
- $n!$ = the product of all integers up to n (thus $186!$ is the product of all integers from 1 to 186)

This formula forms the basis for further formulæ which I have developed from it (notably number (4), given below), and it is indeed the basic formula for the greater part of our work. It may be employed to determine directly either the probability of any given number of pairs among those drawn, or of any given number of pairs among those not drawn.

In a concrete case the formula works out as follows: Suppose that out of 20 individuals (10 pairs), 9 individuals are drawn. What is the probability that there will be just 3 pairs included among those drawn?

Here $m = 20$; $n = 9$; $k = 3$. The formula (3) therefore becomes

$$x = \frac{9!11!10!2^3}{20!3!3!4!}$$

which gives $x = 0.2005$, so that there is almost exactly one chance in five of getting 3 pairs when 9 are drawn from 20. We may put the question in the reverse way by asking: What is the probability of there being left 4 pairs when 11 are left out of 20? (If 9 are drawn from 20, and 6 of the 9 from 3 pairs, then the other 3 belong to other pairs, so that 6 pairs are represented among those drawn, leaving 4 complete pairs among those not drawn). In this case m is 20, n is 11, while k is 4, and formula (3) therefore becomes

$$x = \frac{11!9!10!2^3}{20!4!3!3!}$$

which is identical with the formula (given above) for 3 pairs when 9 are drawn from 20. Thus, the probability of the number of pairs actually drawn is of course bound to be the same as that for the number of pairs actually left, as was previously mentioned.

Determining by formula (3) the probability that there should be 38 pairs among the 103 left from the 186 lines in Miss Cull's case, above cited, we find this to be but 0.00004556; or the odds against this number are 21,948 to 1.

The result at this stage however does not give us what we need to know. If 21,949 tickets were placed in a box and one of these drawn out, the odds would be 21,948 to 1 against any particular number, yet some particular number would be drawn, and there would be no ground for surprise that it should be one particular number rather than another. Our present case is not entirely similar to this, since a certain number (18) of pairs is more probable than any other. Yet formula (3) shows that the probability for this most probable number is but 0.16685, so that this precise number of pairs would be found in but one case out of every 6, and for any given case the odds against it are 5 to 1.

What we require to know is, not only what is the most probable number of pairs, and the deviation from this most probable number, but (and this is the essential point): How probable is it that there should be so great a deviation from this most probable number as that which we find?

Thus, in Miss Cull's case already cited, the most probable number of surviving pairs we know to be 28, while the actual number surviving is 38, so that the observed number deviates by 10 from the most probable number. We require to know how probable it is that there should be so high a deviation as 10.

The probability of a deviation so great as that observed may be determined directly as follows: Determine the probability of all cases showing a deviation less than the given deviation and compare the sum of these probabilities with the sum of all cases showing a deviation as great as the given deviation. In Miss Cull's case, where the most probable number of surviving pairs is 28 and the deviation is 10, we should have to find the sum of the probabilities for all numbers deviating less than 10 (that is, of all numbers from 19 to 37), and compare this with the sum for all numbers deviating more than 10 (all numbers below 19 and above 37).

Since the sum of all the probabilities is 1, it is practically only necessary to find the probabilities for all numbers showing less than the given deviation (in this case, for all numbers from 19 to 37) and subtract their sum from 1; the remainder will be the sum of the probabilities for all deviations as great as (or greater than) the given deviation. This procedure is that which in the Appendix is formulated as rule (5).

For finding the probabilities for a series of successive numbers (as 19 to 37, in the case above), the following formula (4) will be found convenient. First find the probability by formula (3) for the lowest number of pairs (in this case 19). Call the probability for this number x_1 . Then the probability for the next higher number of pairs (which we may call k_2 ; in this case k_2 is 20) is given by multiplying the probability x_1 by

$$\frac{(n - 2k_2 + 1)(n - 2k_2 + 2)}{4k_2(1 - n + k_2)} \quad (4)$$

The probability for the next higher number of pairs (in this case 21) may then be found from this result by using the same formula anew, and so on for the entire series of numbers. (Or if we desire we may begin with the higher numbers, and find the probability for succeeding lower numbers by using the expression (4) inverted.)

An example may be taken. In the case we have cited, we find that the probability for 19 pairs is, by formula (3), 0.000059733. To find the probability for 20 pairs we must multiply this value, according to formula (4), by $\frac{64 \times 65}{4 \times 20 \times 10}$, which gives 0.00031061 as the probability for 20 pairs. To find the probability for 21 pairs we must now multiply this value by $\frac{62 \times 63}{4 \times 21 \times 11}$, giving 0.001313 as the probability for 21 pairs.

Proceeding in this way, and adding together the value of the probabilities obtained, we find that the total probability for all numbers from 19 to 37 is 0.99995242; this then is the probability that the deviation shall be less than the observed deviation 10, if the distribution of deaths has no relation to the pairing. The

difference between this value and 1 gives 0.00004758 as the probability that we should have any deviation as great as 9. Dividing the former value by the latter, we find that the odds against so great a deviation as that actually observed are 21,016 to 1, unless there is something in the pairing that causes the two members of the pairs to be more alike in their fate (not less alike, as the theory of sexual differentiation held).

It would be of interest and value if it were possible to find for the present case some standard of value corresponding to the probable error or standard deviation of the common 'normal' curve of probabilities. It may be of interest to examine a curve for such cases as those we are here dealing with. To get such a curve, we must determine the probabilities for each of the possible numbers of pairs (in the way just set forth); then these may be plotted on some convenient scale. In doing this, one or two simple considerations will aid. If the number drawn is less than half of the total number (that is, if n is less than $\frac{1}{2}m$), then evidently it is possible that all the numbers drawn should be different, so that we must begin with 0 pairs. Further, the number of pairs might be $\frac{n}{2}$ (or $\frac{n-1}{2}$, if n is odd), but cannot be greater than this. So in such a case we must find the probability for each number of pairs from 0 to $\frac{n}{2}$.

I have plotted such a curve for one of the cases described by Miss Cull. She found that after twenty days, 51 lines had died out, from the entire 186; and among these were 13 pairs. In this case the possible numbers of pairs range, in accordance with the considerations just adduced, from 0 to 25.

Determining by formulas (3) and (4) the probability for all these numbers, we obtain the results given in table 36, page 412. We employ these probabilities as ordinates, while the numbers of pairs are laid off on the abscissa. This gives a curve or polygon such as is shown in figure 1. The sum of all the ordinates is here equal to 1 (or if we prefer we may take the total area of the polygon as 1). It is evident that this polygon bears some resemblance to that obtained from distributions following the normal law, but

differs from it; particularly in the fact that its upper limit is much farther from the mode than is the lower limit. This is not characteristic of all polygons obtained in this way, but apparently so only of cases where m and n are rather large. In many cases we have, in place of such a polygon, merely a point, a straight line, or a broken line, which may be of various forms. Some of these are illustrated in figure 2.

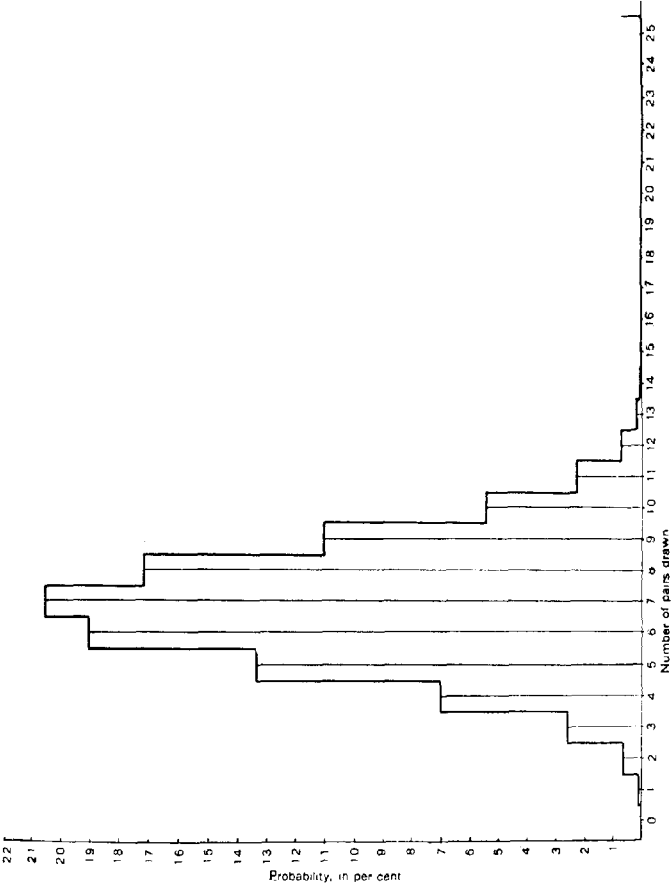
In view of the form of the polygons obtained, it would appear to be difficult to obtain any simple formula to express the probability of a given deviation: one must use such a method as that set forth above (see rule (5), Appendix).

The procedure which we have just described of course makes it possible to determine with absolute accuracy what is the most probable number of pairs when an odd number of units is drawn; a point which we left undecided in our previous account. When this is the only question to be answered, it is done as follows: Find by formula (1) the average number of pairs to be obtained when the given odd number is drawn. Then find by formulae (3) and (4) the probabilities for the two numbers nearest this average; this will of course show which of the two is the most probable.

Formula (3) gives unmanageable numbers when the operations indicated are directly performed; thus $186!$ gives a number consisting of 343 integers. In practical work therefore logarithms must be employed. The logarithm for any factorial number $n!$ is of course the sum of the logarithms of all integral numbers from 1 up to and including n (since $n!$ is the product of all numbers from 1 to n). With a table of such sums of logarithms the com-

Fig. 1 Polygon showing the relative probabilities for obtaining the different possible numbers of pairs, when 51 specimens are drawn from 186 (93 pairs). The ordinates give the probabilities in per cent, for each of the numbers of pairs indicated on the base line. (These probabilities are the percentage of all drawings in which the given number of pairs would be obtained, if the drawing of 51 from 186 were repeated a great number of times.) They are plotted from the data given in table 36, page 412.

The polygon extends at the left to 0, at the right to 25 pairs, but the probabilities for 0 and for all numbers from 15 to 25 pairs are so minute that the ordinates do not appear at all in a polygon drawn to this scale, so that in these regions the outline of the polygon as drawn coincides with the base line.



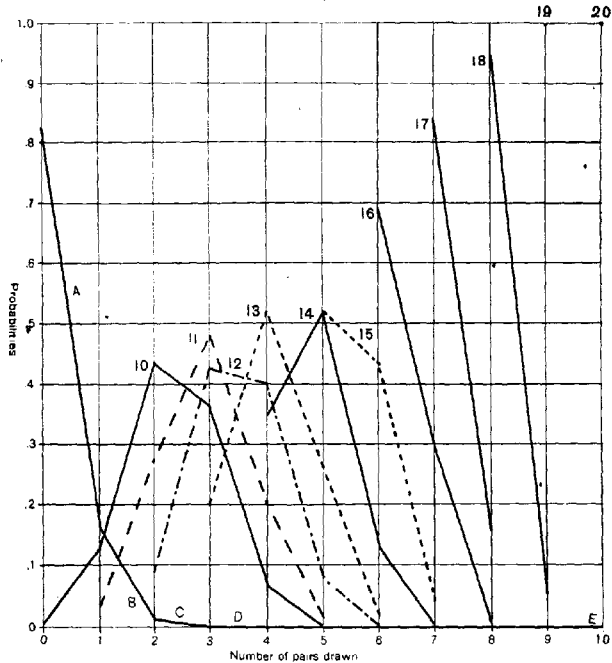


Fig. 2 Examples of the form of graphs for the distribution of the probabilities of the various possible numbers of pairs, in certain cases. The figure is designed to illustrate the variety of forms of such graphs, and to show how little they resemble, in many cases, the 'normal curve.' The ordinates are probabilities; the abscissas are numbers of pairs, from 1 to 10. The graphs are drawn, like the usual polygons of variation, by connecting the tops of the ordinates representing the probabilities.

The line A-B-C-D-E shows the probabilities for the different possible numbers of pairs obtainable when 20 specimens are drawn from 1000 (500 pairs). From 3 up to 10 pairs (D to E) the probabilities are so small that they do not show in a figure drawn to this scale, so that the line representing them coincides practically with the 0 line.

The remaining graphs (numbered 10 to 20) show all those obtainable when any possible number of specimens is drawn from 20 (10 pairs). The numbers adjacent to the graphs show the number of specimens drawn; thus the graph numbered 10 shows the probabilities for the various possible numbers of pairs (0 to 5) when 10 specimens are drawn from 20. As the figure shows, the graphs for 17 and 18 are straight lines; those for 19 and 20 are points.

These graphs (10 to 20) are identical with those to be obtained when the numbers 0 to 10 are drawn from 20, save that in the latter case: (a) they stand in the reverse order, the graph for 1 being the same as that for 19, the graph for 2 the same as that for 18, etc.; (b) all would begin at the left on the 0 line, so that they would be crowded together. Otherwise they have the same form, slope, and dimensions as those shown, for drawing 10 to 20.

putations become fairly simple. Such tables have been published by De Morgan ('45) (six-place logarithms), and by Degen ('23) (twelve place logarithms of $n!$ for all numbers from 1 to 1200), but I have not been able to obtain these. Pearl and McPheters ('11) have recently published a useful table of such sums of logarithms for the numbers from 1 to 100. This table will be found convenient when the numbers dealt with fall within it. For use in the present investigation I have prepared such a table up to 243!.

But the value of $n!$ for any number may be found with sufficient accuracy from Stirling's formula. This, in form for practical use, is as follows:

$$n! = \frac{n^n}{e^n} \sqrt{2\pi n}$$

Here:

$$\begin{aligned} e &= 2.7182818 \text{ (log. } 0.4342944819) \\ \pi &= 3.1415927 \text{ (log. } 0.4971498726) \\ \sqrt{2\pi} &= 2.506628 \text{ (log. } 0.3990899342) \end{aligned}$$

All the operations should be performed by the aid of logarithms (even where a computing machine is available), the best course of procedure being as follows:

- (1) Find the logarithm of n ; multiply this by n .
- (2) Multiply log. 0.4342944819 by n , and subtract this product from the result of (1).
- (3) To the result of (2) add log. 0.3990899342.
- (4) To the result of (3) add $\frac{1}{2}$ the logarithm of n .

This gives the logarithm of the given number $n!$, which may be used in formula (3). For example

$$186! = \frac{186^{186}}{e^{186}} \sqrt{2\pi} \sqrt{186} = \log. 342.8844688$$

In using formula (3) it is of course necessary merely to add together the logarithms of the factors in the numerator, and subtract from the result those of the denominator; the result is the logarithm of the probability.

ANALYSIS OF OBSERVED RESULTS

The view that there is a sexual differentiation between the two members of pairs has been based upon two sets of facts: (1) upon the distribution of survivals and deaths among the ex-conjugants; (2) upon differences in the rate of fission between the progeny of the two members of pairs. We shall deal with these separately, taking up first the deaths.

DISTRIBUTIONS OF SURVIVALS AND DEATHS AMONG THE MEMBERS OF PAIRS

Miss Cull's experiments

We may analyze first the data given by Miss Cull ('07). Ninety-three pairs of conjugants, giving 186 separate lines, were isolated; each line was kept in a vial. At the end of seven days 32 of the lines had died out, including both members of pairs in 6 cases (that is, '6 entire pairs'). After twenty days 51 of the lines had died out, including 13 pairs. After 30 days 83 lines had died out, including 28 pairs. We have then three cases to deal with. Is the number of complete pairs dead in any or all of these cases less than would be expected if the distribution of deaths had no relations to the pairing, as would be required if this is to be considered evidence for sexual differentiation? Or, what is the same thing, is the number of pairs surviving less than would be expected if the distribution of deaths had no relation to the pairing? We need to deal explicitly with but one of these two questions, since the answer is absolutely identical in the two cases. As a rule therefore we shall take up the question only in the form first stated.

First case. Here, after seven days

$$\begin{aligned} m &= 186 \\ n &= 32 \\ \text{number of pairs dead} &= 6 \end{aligned}$$

Now, applying our formula (1) or (2), we find that the most probable number of pairs, if the distribution of deaths is random, is 2, although 3 is nearly as probable. The average number

of pairs that will be obtained if successive drawings are made is by formula (1), 2.68. Five actual drawings of 32 tickets out of 186 gave respectively 4, 2, 1, 2, 4 pairs; the average being thus 2.6; very close to what the theory calls for.

The observed number 6 is therefore greater than would be expected, not less, as the theory of sexual differentiation would expect. By formula 3, the probability for 2 pairs is found to be 0.27233; that for 3 is 0.26808; for 6 is 0.02013. The observed number (6) deviates from the most probable number (2) by 4; we require to know what is the probability that there should be so great a deviation as this. By our rule (5) (Appendix), we find that the total probability for a deviation as great as 4 is 0.0248, while for deviations less than 4 it is 0.9752, so that the chance is 39.3 to 1 against there being a deviation so great as that observed, if the distribution of deaths is independent of the pairing.

Thus, so far as this case goes the probability is very strong that the distribution of deaths is not independent of the pairing. But its dependence is of a character the reverse of what the theory of sexual differentiation requires; the number of pairs is greater, not less, than we should expect from a random distribution of deaths. Thus it appears that the members of the pairs are more alike, not less alike, in this respect, than would be anticipated. The drawing of further conclusions may be deferred until other cases are examined.

Second case. After twenty days, Miss Cull found that 51 lines had died, out of 186, and among these were 13 pairs.

By formula (1) the most probable number of pairs is found to be 7, the *average* number if a great number of drawings were made being 6.892. Five drawings of 51 tickets out of 186 gave respectively 6, 9, 8, 8, 8 pairs, the average being 7.8, which is very close to theory; the excess of nearly 1 showing what may happen when but few drawings are made.

The observed number of 13 pairs exceeds the most probable number by 6 pairs. What is the probability that there should be so great a deviation as this from the most probable number of pairs?

In this case I have worked out by formula (3) the value of the probability for all possible numbers of pairs; the results are given in table 36.

From this table is plotted the curve of figure 1 (page 407). The curve shows directly to the eye how great is the divergence of the observed result from what should be expected if the distribution of deaths has no relation to the mating; the ordinate of 13, the actual number of pairs, is far from that of the most probable number of pairs. By our rule (5) we find that the probability of so great a deviation as 6 from the most probable number is

TABLE 36

Probabilities for all possible numbers of pairs, in the case where 51 individuals die out of a total of 186.

NUM- BER OF PAIRS	PROBABILITY
0	.0000676
1	.0009644
2	.0064441
3	.0258003
4	.069407
5	.133352
6	.189841
7	.205061
8	.170717
9	.110648
10	.056170
11	.022402
12	.007018
13	.001722
14	.000329
15	.0000487
16	.0000055
17	.0000005
18	.00000003
19	.000000001
20	.00000000004
21	.0000000000009
22	.00000000000001
23	.0000000000000008
24	.00000000000000002
25	.0000000000000000002

0.00224, while the probability that the deviation will be less than this is 0.99776. Hence the chances are 445 to 1 against a deviation so great as is actually observed, if the distribution of deaths is independent of the pairing.

Thus it appears that as further deaths take place, the tendency for both members of a pair to die, if one dies, becomes greater; after seven days the chances were but 39 to one against so great a deviation as occurs, while after twenty days the chances against the actual result are more than ten times as great as they were. It is therefore clear that among those who died in the interval between the seventh and the twentieth days there were a greater number of mates of those that had previously died, than could be expected as a matter of chance. This is clear when the exact figures are considered. At the end of seven days there were left 154 individuals, including 134 that were still paired, while 20 were odd (their mates dead). Now, in the next thirteen days there died 19 more, and of these 19, no less than 14 were mates of those that had already died (or that died during the entire twenty days), while but 5 fall to the 134 paired individuals! It is clear that whatever is causing the death of one individual of a pair has a strong tendency to cause likewise the death of its mates; the mates are much more alike in this respect than are two lines taken at random. And in accordance with what has previously been set forth, we may make the same statement for the survivals. Whatever tends to cause one individual of a pair to survive has likewise a strong tendency to cause the survival of its mate—the numerical relations as to survival being identical with those above set forth for the deaths.

Third case. After thirty days Miss Cull found that out of the 186 lines, 83 had died out, including 28 pairs. We have already dealt with this case, but may here recapitulate the facts. By our formula (1), the average number of pairs obtained when 83 are drawn from 186 is 18.39, from whence it may be concluded that the most probable number of pairs is 18. The excess over the most probable number has therefore now grown to 10 pairs, though at the end of 20 days the excess was but 6 pairs. This

shows that the tendency for mates to die continues into this third ten-day period. At the end of the second period (twenty days) there had been left 110 paired lines and 25 odd ones. There die in the next ten-day period 32 additional lines, and of these, no less than 30 are mates of those already dead, or of those that die during the present period, while but 2 are mates of those that still live at the end of this period!

As one would expect, the chances for such a result, if the distribution of deaths has no relation to the pairing, is infinitesimally small. By formula (3), we find that the chance for getting 28 pairs is but 0.0000455623. The chance for getting any number whatsoever that deviates from the most probable number (18) as much as does 28 is almost equally small. By our rule (5) we find that the probability that the deviation from 18 will be less than 10 (the observed deviation) is 0.999952421, while the chance that it will be as much as 10 is but 0.00004758. Thus the chances are 21,016 to 1 against there being so great a deviation as was observed, unless the distribution of deaths depends upon the pairing.

Thus a more complete demonstration that the distribution of deaths depends on the pairing could not be demanded, but the relation is throughout the reverse of that called for by the theory of sexual differentiation. There is not a tendency for one member of the pair to live and for the other to die; on the contrary, there is a most marked tendency for both members to have the same fate. The effects of this tendency show themselves more and more strongly as the lines of progeny successively die out after the pairing. Supposing that such a tendency does not exist, the chances, after seven days, are 39 to 1 against getting so great a deviation as exists; after twenty days the odds are 445 to 1; after thirty days they are 21,016 to 1.

It appears from the figures given by Miss Cull that there is something in the process of pairing that causes the members of pairs to become alike, so far as survival and death are concerned. This will now be tested by other experiments and in other relations.

Calkins' experiments

Calkins in his paper of 1902 gives some statistics bearing on this matter. In his table 3 he gives records for the progeny of 40 pairs of conjugants, 24 being 'exogamous,' 16 'endogamous.' These were not all taken nor cultivated at the same time, so that it is doubtful how far we should attempt to use them for our present purposes. However, some of the facts may be given, in order to show any relation they may have to our problem. Calkins notes that a large proportion of the lines die out before the tenth day. We may then select this period for examination. It is found from Calkins' table that by the end of the tenth day 40 of the 80 lines derived from the 40 pairs have died out. Here we have $m = 80$; $n = 40$. The most probable number of pairs is found, by our formula (1) or (2), to be 10. The actual number of pairs dead is 14. Thus in this case, as in those described by Miss Cull, the number of pairs dead, and the number of pairs living, is greater than would be expected; not less, as the theory of sexual differentiation demands.

At the end of one month but five lines were living, out of the 80. Among those living there were no pairs; this was to be expected on any theory.

The data given by Calkins perhaps hardly warrant farther analysis for our present purposes.

Experiments of the author

In my paper on the Effect of Conjugation in Paramecium ('13), which immediately precedes the present one, I have given an account of a number of experiments which yield data for attacking our present problem. I will here analyze these data, referring to the paper just mentioned for all details not bearing directly upon the question now in hand. I shall refer to the experiments by the numbers given to them in the paper cited.

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Experiment 1: Paramecium caudatum

This Journal, volume 14, 1913, page 286

In Experiment 1 there were 31 pairs under propagation; one member of one of the pairs was accidentally lost, so that for our present purposes this pair must be excluded from consideration. We have thus 30 pairs to deal with. None of them died during the first week, showing that no deaths were due to handling or the like while the pairs were united. The facts are given in table 29 of the paper just referred to (this Journal, page 379).

It will be well to examine the condition of affairs at certain intervals.

At the end of the second week, five lines had died out, including one pair. By formulae (1), (2) and (3), it is found that the most probable number of pairs is 0, and that the chances are 5.06 to 1 against any pairs whatsoever being included. That is, the chance for 0 is 5.06 times as great as for all other possibilities together.

At the end of the third week 15 lines out of the 60 were dead, and among these fifteen were 4 pairs. Formula (1) shows that the most probable number of pairs is 2; and formula (3) shows that 2 pairs will occur in somewhat more than one third of all cases, while 4 pairs would occur but once in 25 cases, if the distribution of deaths had no relation to the pairing. The deviation of the actual number from the most probable number is 2: by our rule (5), we find that the chances are 5.96 to 1 against so great a deviation as this, unless the distribution of deaths is actually connected with the pairing.

At the end of the fourth week there are 18 lines dead, including 5 pairs. The most probable number is 3 pairs, which would occur in 31 per cent of all cases, while 5 would occur in but 4 per cent, if the distribution is random. The deviation from the expected number has not increased during the fourth week.

At the end of the experiment, five weeks from the beginning, 22 lines were dead, including 5 pairs, so that the number of pairs has not increased. As set forth in the account of this experiment in the paper referred to (page 287), the deaths during the fifth week were due to mistaken cultural treatment; their distribution

had no relation to the pairing. The most probable number of pairs when 22 lines die is 4; the actual number 5. The probability for four is 0.306; for 5 it is 0.214, or about two-thirds as great as for 4. Five pairs might therefore well occur purely as a matter of chance distribution.

Thus in this experiment the number of pairs included among those that died is a little greater than would be expected if the deaths had no relation to the pairing. The relation is again the reverse of that which the theory of sexuality requires.

Experiment 2: Paramecium caudatum

This Journal, vol. 14, 1913, page 293

In this experiment, lasting for eight weeks, 56 lines derived from members of pairs, 38 from split pairs, and 58 from unpaired individuals, were cultivated. The designations of the unpaired lines were arbitrarily grouped in pairs at the beginning, in order to discover whether such arbitrary grouping would give the same relations between its members as appear in the actual pairs.

The death rate was very high in this experiment, for reasons set forth in the preceding paper (p. 293); on this account it does not furnish the best of data for determining whether there is any relation between liability to death and the pairing. It will be worth while however to set forth the facts in the case; this is done in table . I have included the facts, not only as to the pairs, but as to the split pairs, and the free specimens. In the split pairs, the two individuals that had begun pairing are called a pair. In the free specimens, the pairing is purely artificial; these are for control purposes.

As the table shows, in no case does the actual number of pairs dead differ from the most probable number by more than a single one. This is precisely such a result as might be expected in chance single cases. The deviations from the number that is absolutely the most probable one occur, as will be seen, in the case of the free specimens, in which the pairing is purely arbitrary, as well as in the other classes. I have given in the table the

TABLE 37
Death rate in relation to pairing, in experiment 2.

	WHOLE NUMBER OF LINES	DIED	PAIRS DIED	MOST PROBABLE NUMBER OF PAIRS	PROBA- BILITY OF THE ACTUAL NUMBER	PROBA- BILITY OF THE MOST PROBABLE NUMBER
First week:						
Pairs.....	56	7	0	0	0.654	0.654
Split pairs....	38	2	0	0	0.973	0.973
Free.....	58	4	0	0	0.856	0.856
First and second weeks:						
Pairs.....	56	20	2	3	0.168	0.534
Split pairs.....	38	17	4	4	0.345	0.345
Free.....	58	28	6	7	0.263	0.281
Eight weeks:						
Pairs.....	48	39	17	16	0.139	0.449
Split pairs.....	26	20	8	7	0.447	0.477
Free.....	46	35	13	13	0.439	0.439

probability for the occurrence of the most probable number, as well as of the actual number, in order that these may be compared.

Thus the data of this experiment give no evidence for sexual differentiation, nor for any other special relation of the two members of pairs; they are what might be expected if the distribution of deaths were purely random. This is not surprising, in view of the high mortality; the deaths were evidently due mainly to extrinsic causes.

Experiment 3: Paramecium caudatum

This Journal, volume 14, 1913, page 300

In this experiment there were 46 lines, derived from 23 pairs. Out of these, 11 lines died out during the four days of the experiment. The number of pairs among these was 1, and this is the most probable number that would be found, if the incidence of death had no relation to the pairing.

There were also in this experiment 50 lines derived from 25 split pairs; of these 35 lines died out in the four days. Among these were 13 split pairs. Applying our formulae, we find that the most probable number of pairs is 12, although 13 is nearly as probable. The probability for 12 is 0.369; for 13 it is 0.260, so that 13 would occur little less often than 12.

The mortality in this experiment gives then no indication of sexual differentiation, nor of any other special relation between the members of pairs. The very high mortality, taken in connection with the conditions of the experiment, shows that the causes of death were mainly extrinsic.

Experiment 4: Paramecium aurelia

This Journal, volume 14, 1913, page 304

In this experiment with the race *k*, there were included 40 lines derived from 20 complete pairs (2 other lines having lost their mates through accident). Of these, 23 lines died out during the twenty days of the experiment, and among these were both members of 8 pairs. The most probable number of pairs when 23 die out of 40, is 6, if the deaths have no relation to the pairing, although 7 is nearly as probable. Thus the number of pairs that died is greater than would be expected if the deaths had no relation to the pairing; not less, as would be required in order to give evidence of sexual differentiation. The excess is however small, the probability of getting eight pairs being 0.144, so that this would happen in one case out of seven, purely as a matter of chance. No positive conclusions could therefore be drawn from this result, taken by itself.

Experiment 5: Paramecium aurelia

This Journal, volume 14, 1913, page 308

In this experiment on the pure strain *k*, lasting six days, 8 lines died, out of 16 (derived from 8 pairs). Among these were both members of 3 pairs. The most probable number of pairs, if the deaths are distributed at random, is 2. The chance of 2 is 0.522; that of 3 is 0.174.

Among the 9 lines that died from among 20 derived from 10 split pairs, there were both members of 2 split pairs. The most probable number, on a chance distribution of deaths, is likewise 2.

Thus the results in this experiment are, so far as they go, against the hypothesis of sexual differentiation, but do not give definite positive evidence of any other relation between the members of pairs.

Experiment 13: Paramecium aurelia

This Journal, volume 14, 1913, page 329

In this experiment, carried on with the pure strain *k*, there were 78 lines derived from 39 pairs of ex-conjugants; these were cultivated for only a week. During this time there died out 20 lines, including 4 pairs. The most probable number of pairs, with a distribution of deaths having no relation to the pairing, is 2. The probability of 4 pairs is 0.142, so that they would occur, as a result of chance, once in seven cases.

Thus the number of pairs that died is here, as usual, greater than would be expected if the distribution of deaths had no relation to the pairing.

Summary: on the relation of the survivals and deaths to pairing

In order to give evidence of sexual differentiation between the members of pairs, it is necessary that the number of cases in which both members of pairs die or survive should be less than would be expected if the distribution of deaths had no relation to the pairing. In no case, among those of which statistics are available, as set forth in the preceding, is this the state of affairs. The nearest approach to it is in my experiment 2 (page 417), where after two weeks the number of pairs dead was one more than the most probable number, though at the end of the experiment the number dead was one less than the most probable number. In all other cases the number of pairs that died and the number that survived, was equal to, or greater than, the number that would be expected if the distribution of deaths had no relation to the pairing. In most cases it is greater than would be expected.

Thus the distribution of survivals and deaths gives no evidence of sexual differentiation between the members of pairs.

On the contrary, the fact that the number of pairs that survive (or of those that die) is as a rule greater than would be expected as a result of a random distribution of deaths indicates that the two members of pairs are *more* alike than individuals taken at random; that there is thus in the pairing something that tends to make the two members have a similar fate.

This second conclusion could not be drawn with any great degree of confidence from my own experiments, summarized in the preceding section. In most of these the number of pairs was indeed greater than would be expected, but the excess was usually not so great but that it would occur once out of seven or eight cases purely as a matter of chance. It is Miss Cull's experiment that thus far gives the only incontrovertible evidence for something causing a like fate in the members of pairs.

Miss Cull's experiment is of course much better adapted for testing this particular matter than are my own, or those of Calkins. In my experiments the animals were cultivated on slides, for determining the rate of reproduction, so that it was possible to keep only a small number of representatives of each line. If these few died, the line was extinct. In Miss Cull's experiment, however, all the progeny of a given line were retained, in small mass cultures. They were thus practically assured against accidental extinction; if any died out, it indicated some intrinsic weakness. In Miss Cull's experiment, as we have seen, the number of complete pairs that died, and the number that survived, was enormously greater than would be expected; so that the result cannot be attributed to chance. Particularly in the latter part of the experiment does this appear, so that the pairs tend to become grouped into two classes, in one of which both members die out, while in the other both members live. The chances are 21,000 to 1 against the result actually reached, if there is not something in the pairing tending to give the two members a similar fate; in other words the chances are 21,000 to 1 that the latter is the case.

In Part II of this paper further evidence will be given on this point.

COMPARATIVE RATE OF REPRODUCTION IN THE TWO MEMBERS
OF PAIRS*Method of analysis*

The second ground for holding the two members of pairs to be sexually differentiated is the fact that often one of them is vigorous and reproduces freely, while the other is weak and reproduces slowly.

Here arises a question parallel to that which we have dealt with in the preceding section. Differences in vigor and rate of fission are common among the exconjugants, and are of course found likewise between individuals that are not members of pairs. In order to be considered evidence for sexual differentiation, *the differences between the members of pairs must be more marked than the average differences between individuals taken at random.* Otherwise they can only be considered instances of the variability in this respect among the members of a population.

It is true that organisms might be sexually differentiated in other respects, and still the members of pairs might show in this particular respect differences not greater than the average differences between members of the population. But in the present case it is this difference alone that is the ground for the supposed sexual differentiation (particularly since we have shown above that the relative mortality gives no such ground). Unless therefore the difference is more marked than the average differences between individuals taken at random, no ground exists for holding that the two members of the pairs are sexually differentiated. The mere fact that there is variation in rate of fission, distributed without relation to pairing, would certainly be no indication of sexual differentiation.

We may therefore proceed to determine whether the differences in this respect between the two members of pairs are greater than the average difference between individuals.

One special point will be of interest. If the two members of pairs are sexually differentiated, then the one that reproduces most rapidly will have to be taken as representing the female, and the other as representing the male. It will then be possible

for us to determine the average differences between the individuals representing the same sex; between the diverse 'females,' and between the diverse 'males.' If the differences between the two members of pairs are not distinctly greater than those between members of admittedly the same sex, then a fortiori these would constitute no basis for holding the two members to be sexually different.

With these considerations in mind we may examine the data.

Miss Cull's experiment

In Miss Cull's experiment it was found that there is in most cases a difference between the two ex-conjugants with respect to vigor and reproductive power.

At the end of the month, of the 65 pairs then represented by living cells, in twenty-seven pairs, or 41 per cent, one of the exconjugants only, or the off-spring from it were alive; in fifteen pairs, or 23 per cent, the progeny of one exconjugant was three times as large as that of the other; in six pairs, the descendants of the one were twice as numerous as those of the other organism; and in only five cases had both conjugants given rise to the same number of offspring. The twelve remaining pairs showed a wide diversity in the number of paramecia produced by any two conjugants. (Cull '07, p. 88).

The differences were determined by examining the number of progeny present in the vial culture at the end of the month.

It needs to be remembered that a single additional fission doubles the number of progeny in one conjugant as compared with the other; so that a doubling of the number of progeny by no means signifies a large difference in the rate of fission, in the course of a month, or even in the course of a few days. Such a difference in the number of progeny might be produced even though the rate of fission in the two were only slightly different. Now, we have no further statistics on this case, for determining how far there are variations in the rate of fission even when we take the organisms quite at random, so that we cannot tell how the differences between the members of pairs compare with the differences we should find if we took two individuals at random.

Experiments by the author

In my Experiment 1 (this Journal, vol. 14, 1913, page 286) we have the data for determining this point. In this experiment there were 120 lines derived from animals that began conjugation; 61 from those that were allowed to complete the process, 59 from those that were separated before the union was consummated (split pairs). In the five weeks during which the experiment was continued, none of the strains from the split pairs died out, showing that any deaths among the progeny of the pairs were not due to lack of a revivifying effect of conjugation. We have already dealt with the deaths among the conjugants (page 416). Here we will deal with the differences between members of pairs in the rate of reproduction.

The number of fissions for five successive weeks are given for the progeny of each member of the pairs in table 29 of the paper on the effects of conjugation (this Journal, vol. 14, 1913, page 379). From this table we may find the average difference between members of pairs in number of fissions for any given period; and also the average difference that would exist if the organisms were paired at random (that is, the average difference between any two individuals of the lot).

The method by which this is done may be illustrated as follows, taking the fissions for the first week as an example. Here the fissions are distributed among the 60 members of pairs as shown in the following table 38 (data taken from table 29 of the paper on the effects of conjugation '13).

The differences in number of fissions between the two members *a* and *b* of the 30 pairs are distributed as shown in the following table 39 (data from table 29 of the preceding paper).

TABLE 38

	FISSIONS						
	0	1	2	3	4	5	6
Most rapid member of pairs (<i>a</i>).....	1	1	1	3	11	11	2
Slowest member of pairs (<i>b</i>).....	5	5	2	7	8	3	
Total.....	6	6	3	10	19	14	2

TABLE 39

		DIFFERENCES IN NUMBER OF FISSIONS					
		0	1	2	3	4	5
Number of pairs.....	12	5	5	2	5		1

From table 39 it is found that the average difference between the two members of the actual pairs is for the first week 1.533. To find the average difference between any two members (or the difference that would exist if the animals were paired at random), we must pair each member with every other. It will be found that for any given number n of organisms, the number of diverse pairings that can be made is $1 + 2 + 3 + \dots$ up to one less than the given number; this is equivalent to the expression $\frac{1}{2} n (n - 1)$. Thus, for the 60 organisms of the first week, the number of possible pairs is $\frac{1}{2} (60 \times 59) = 1770$.

To make all possible pairings we proceed as follows, taking the 'totals' of table 38 as an example: Arrange a table for the possible differences, as in the first row of table 40. Now, in table

TABLE 40

		DIFFERENCES						
		0	1	2	3	4	5	6
Number of pairings.....	15	36	18	60	114	84		12
	15	18	60	114	84	12		
	3	30	57	42	6			
	45	190	140	20				
	171	266	38					
	91	28						
		1						
Total pairings.....	341	568	313	236	204	96		12 = 1770

38 there are 6 members showing 0 fissions. If these 6 are paired among themselves, the differences between them will likewise be 0. The number of diverse pairings among the 6 is given by the formula $\frac{1}{2} n (n - 1)$; for $n = 6$ this gives 15 pairs. We enter this 15 beneath the 0 of table 40.

The 6 members at 0 fissions must likewise be paired with the 6 at 1 fission, giving 36 in which the difference is 1; we thus enter this 36 beneath the difference 1, in table 40. In the same way we successively pair the 6 (at 0 fissions) with all the other totals of table 38, entering the products under the proper differences in table 40; this gives the first row of 'number of pairings' in this table.

We proceed in the same way with the remainder, each 'total' of table 38 giving under the difference 0 the entry found by the formula $\frac{1}{2} n (n - 1)$, and successively under the other differences the product of this total with each of those following it. This gives, for the entire first week, table 40. We next sum the number of pairings for each difference, as in the last row, and get their total sum (1770). A control for the accuracy of the work up to this point is given by the fact that this total sum must be equal to $\frac{1}{2} n (n - 1)$, where n is the total number of diverse lines (in this case 60).

Next we multiply each difference of table 40 by the sum of the 'total pairings' under it, and add these products, giving the total sum of differences for all the pairings. In the present case this total sum is 3270. Dividing this by the total number of pairings (1770), we find that the average difference between members, when all possible matings are made, is 1.8474.

In the same way we may if we desire find the average difference between the most rapidly dividing members ('females') of pairs; and between the slowest members ('males').

Carrying out these operations for the five weeks of Experiment 1 (table 29 of the paper on the effect of conjugation, page 379), and for certain combinations of these periods, we obtain the results given in table 41.

The results given in table 41 are surprising. *In every case the difference between the members of actual pairs is not greater, but less, than the average difference between two individuals taken at random.*

Thus we find, for the rate of reproduction, as for the distribution of deaths, and of survivals, that the two individuals do not resemble each other less, but more, than the average individuals.

There is something in the pairing that causes the two individuals to resemble each other more than usual.

The condition is again the reverse of that called for by the theory that the two members of pairs are sexually differentiated. I pointed out on a preceding page the interest of the question as to whether the two members of the pairs differ from each other more than do members of what would have to be considered members of the same sex. I have worked out this matter for a number of periods, for the same organisms dealt with in table 41. The results are given in table 42.

Table 42 shows that the difference between the two members of pairs ('male and female') is on the average distinctly less than the difference between those that would have to be considered members of the same sex in different pairs. It is less than the difference between the 'females' of table 42 in two of the four weeks of table 42, greater in two, and averages practically the same. It is decidedly less than the difference between the 'males' of different pairs, in three of the four weeks; greater in one; it averages distinctly less than for the 'males.'

Thus, so far as the conditions in experiment 1 are typical, we must conclude that the differences in rate of reproduction

TABLE 41

Experiment 1. (This Journal, vol. 14, 1913, p.286). Mean differences in number of fissions between the two members of pairs, as compared with the mean differences of members paired at random. The table includes only those pairs, both members of which lived through the period in question, save the last entry, which gives the data for the actual number of fissions for all the pairs that began the experiment whether they lived to the end or not.

	NUMBER OF PAIRS	DIFFERENCE	
		Members of actual pairs	Random pairing
First week.....	30	1.533	1.847
Second week.....	26	1.615	2.384
Third week.....	19	2.895	3.060
Fourth week.....	17	1.882	2.474
First two weeks.....	26	2.731	3.728
Four weeks.....	17	5.000	5.966
Five weeks.....	13	6.154	7.246
Five weeks, all pairs.....	30	8.333	10.998

TABLE 42

Experiment 1. Average difference in fission rate between the members of pairs, as compared with the average difference between the 'females' of different pairs; and also between the 'males' of different pairs. The most rapidly reproducing member of a pair is designated 'female'; the slower one 'male'. Based on data of table 29 of the paper on the effect of conjugation, (page 379).

	AVERAGE DIFFERENCE BETWEEN		
	Members of actual pairs	'Females' of diverse pairs	'Males' of diverse pairs
First week.....	1.533	1.349	1.910
Second week.....	1.615	1.902	2.505
Third week.....	2.895	2.363	2.666
Fourth week.....	1.882	2.265	2.059
First 2 weeks.....	2.731	2.748	4.043
Mean for the 4 weeks.....	1.981	1.970	2.285

between the members of pairs cannot be considered evidence of sexual differentiation. For this difference is actually less than that between individuals taken at random; and not more than that between individuals that would, on the sexual theory, have to be considered members of the same sex.

Biparental inheritance?

Thus in studying the question of sexuality, we have come upon a matter that is of much interest quite independently of this question. The fact that the progeny of two individuals which have conjugated resemble each other more than do those of two individuals taken at random would seem to indicate that they must inherit from both the conjugants. If this be the case, we have here the first evidence that has been presented of biparental inheritance in Protozoa, and particularly in connection with that form of conjugation in which both the conjugants continue to reproduce.

Analysis of the experimental results

From this point of view we may examine the facts further. Since in Experiment 1, as we have seen, the two strains derived from a pair resemble each other in rate of reproduction more than

others, we may expect to find that there is a positive coefficient of correlation between them, in this respect. To determine the coefficient of correlation is a more accurate and elegant way of discovering whether the two members of a pair are more alike than strains taken at random, than that which we have thus far employed. I have therefore computed the coefficients of correlation for Experiment 1, and also for other experiments, to be set forth. In these cases we shall as a rule not employ the method thus far used, but consider that the determination of the coefficient of correlation suffices for answering the questions in which we are interested.

In studying the coefficient of correlation in rate of reproduction, we are dealing with integral variates; namely, the number of fissions in a given period; these can be counted exactly. Furthermore, the two members of the pairs are alike, so that we have the condition for which symmetrical correlation tables have been used. For determining the coefficient, I have employed mainly the 'difference method' (Harris '09), which is peculiarly applicable to cases of the present character. The formula for the coefficient of correlation is for such cases by this method

$$r = 1 - \frac{\sigma_v^2}{2\sigma_x^2}$$

where x = the number of fissions, and v = the difference in number of fissions between the two members of a pair.

In this formula it is important to remember that σ_v^2 is given by the sum of the squares of the *positive* differences between the members of pairs, divided by the number of pairs. The formula can be written

$$r = 1 - \frac{\frac{\sum v^2}{n}}{2\sigma_x^2}$$

where n is the number of *pairs*, and v is the positive difference.

In using this formula, it is not necessary to arrange the data in a correlation table, since all that one requires is, the standard deviation of the number of fissions (for all members together), and the standard deviation of the differences between the two members of pairs. These differences can be readily taken directly from such a table as table 29 of the preceding paper on the effects of conjugation (page 379), and arranged at the same time as in table 39 of the present paper.

Our chief data from the work set forth in the preceding paper on the effects of conjugation are from Experiment 1. In the first column of table 43 are given the coefficients of correlation between members of pairs for the various periods of this experiment, as well as certain coefficients from Experiments 2 and 3.

The coefficients from Experiment 1 are based on the data given in table 29 of the preceding paper (page 379); they can be recomputed from that table by anyone desiring to test the work. Besides the coefficients for weekly periods, I have given also those obtained by taking for each member the number of fissions for periods of two weeks, for four weeks, and for five weeks. It is important to understand that these latter are not averages, derived in any way from the coefficients for the separate weeks, but are computed directly from the data as to the number of

TABLE 43

Coefficients of correlation in rate of fission between the two strains derived from the two members, a and b, of pairs, and of split pairs, in experiments 1, 2 and 3 of the preceding paper (Paramecium caudatum). The coefficients for experiment 1 are computed from the data given in table 29 of the preceding paper, page 379.

	PAIRS: CONJUGATION CONSUMMATED		SPLIT PAIRS: CONJUGATION NOT CONSUMMATED	
	Number of pairs	Correlation of a with b	Number of split pairs	Correlation of a with b
Experiment 1:				
First week.....	30	0.1155 ± 0.0859	24	0.2980 ± 0.0887
Second week.....	26	0.4925 ± 0.0708	24	0.3082 ± 0.0881
Third week.....	19	0.1334 ± 0.1074	24	0.2322 ± 0.0921
Fourth week.....	17	0.4355 ± 0.0937	24	0.4936 ± 0.0733
First two weeks.....	26	0.4450 ± 0.0708	24	-0.0686 ± 0.0969
Second two weeks.....	17	0.3377 ± 0.1025	24	0.4362 ± 0.0788
Four weeks.....	17	0.2734 ± 0.1070	24	0.2758 ± 0.0899
Five weeks.....	13	0.3074 ± 0.1108	24	0.1296 ± 0.0957
Four weeks, all pairs.	30	0.4788 ± 0.0671		
Five weeks, all pairs.	30	0.4038 ± 0.0729		
Experiment 2:				
Two weeks.....	10	0.4825 ± 0.1137	6	-0.1800 ± 0.1884
Two weeks, all pairs.	28	0.1027 ± 0.0892		
Experiment 3:				
Four days.....	13	0.4628 ± 0.1030		

fissions in each strain for the period set forth; it is entirely possible that two weeks should when considered separately each give a positive coefficient, while taken as one period they should give a negative one, or vice versa, so that the coefficients for the longer periods furnish independent data. In all cases except the three entries where the words 'all pairs' are added, there is included in each case only the strains in which both members of the pair lived through the period in question. In the three where the words 'all pairs' are added, I have given the coefficient which appears when we include the number of fissions for all the strains that entered the experiment, whether they lived through the period in question or not; taking thus, in the case of Experiment 1, all the 'totals' of table 29 of the preceding paper.

In every case, as table 43 shows, there is a positive coefficient of correlation between the progeny of members of pairs. In one or two cases this is small and would hardly be significant in comparison with its probable error, if it stood alone; but in most cases the coefficient is of very considerable value. The coefficient persists and is even in Experiment 1 increased, when we include the number of fissions up to the time of death in the strains that died before the end of the period in question, as in the last two entries for Experiment 1. This is due to the fact that there is a similarity in the two strains as regards length of life; a point more fully brought out in the study of the distribution of mortality, already made.

The data from Experiments 2 and 3 of the paper on the effects of conjugation are less full than those for Experiment 1, the members of pairs being still smaller. The coefficients are given in table 43 for what they are worth; they confirm so far as they go the results from Experiment 1.

The question which next arises is as to the cause of the fact that, as shown by these positive coefficients, the progeny of the two members of a pair resemble each other in rate of reproduction more than do those of unpaired individuals. Here there are two possibilities:

1. The resemblance may be due to inheritance from both parents. This would fully account for the facts, and appears a highly

probable explanation. The resemblance would in this case be a result of conjugation.

2. The resemblance might be due to assortative mating. We know that assortative mating does occur in *Paramecium*, so far as size is concerned, individuals of nearly equal size tending to mate together (Pearl '07, Jennings '11). It appears possible that the similarity in size might be accompanied by a similarity in physiological characteristics, resulting in similar rates of fission. This would be especially notable if there were a tendency for members of the same or related strains to mate together; and this tendency does exist, as I showed in my paper of 1911. Thus it appears not improbable that the correlation is partly due to assortative mating. So far as this is the correct explanation, the resemblance would exist before the mating took place; would not be a consequence of conjugation.

It seems on the whole probable, *a priori*, that both inheritance and assortative mating play a part in bringing about the greater resemblance in the progeny of the two members of pairs.

How can we test the validity of these explanations? If assortative mating plays a part in the matter, then the resemblance in the two strains derived from a pair ought to exist, to a certain degree at least, if the two members of the pairs are separated before conjugation is consummated. Now, this operation was performed, for other purposes, in Experiments 1 and 2 of the preceding paper. In these cases we had the progeny both of pairs and of 'split pairs.' By determining whether there is correlation between the two individuals, *a* and *b*, of the split pairs (individuals that were uniting for conjugation), we can tell whether assortative mating plays any part in the matter. The coefficients are given in the second column of table 43.

As there appears, there is in fact a positive coefficient of correlation, in most cases, between the progeny of animals that were beginning to pair, but did not complete the process. The correlation is on the whole of similar value to that found between the two members of pairs that had completed conjugation. If we average the eight coefficients for Experiment 1 in each case, we find that the mean for the conjugants is 0.3176; for the non-con-

jugants 0.2635; so that that for the conjugants is about 20 per cent greater.

It would seem therefore that assortative mating accounts for at least a part of the similarity between the progeny of the pairs. Whether any part of it is due to inheritance can hardly be determined from our data up to this point, since the probable errors are in all cases large, owing to the small numbers of pairs in the experiments.

What we need clearly is, data based on larger numbers. Such will be supplied in Part II.

PART II

H. S. JENNINGS AND K. S. LASHLEY

EXPERIMENT 16: 241 PAIRS OF CONJUGANTS PROPAGATED SEPARATELY FOR FORTY-SEVEN DAYS

On the work presented in Part I the criticism may be made that the numbers dealt with are hardly sufficient to place beyond all doubt the conclusions to which they lead. This applies particularly to the results shown in the measurements of the similarity in rate of reproduction between the descendants of the two members of pairs; the coefficients of correlation are variable and their probable errors are large. It hardly applies to the study of the distribution of the deaths among the exconjugants, since here we had the relatively large number of Miss Cull's valuable experiment (93 pairs) to work with, but these results should be tested by others. It is true that the difficulty in dealing with large numbers is very great when the rate of reproduction for each strain must be recorded for long periods. But the fact that it is difficult to work with large numbers does not lessen the insecurity of results drawn from small ones.

Furthermore, all the results thus far have been drawn from experiments designed for other purposes; the question arises as to whether all conditions have been fulfilled for getting accurate results on the present problems. One point in particular suggests itself. In the experiments thus far, the two members *a* and *b*

were cultivated side by side in the two concavities of a single slide. In changing the animals to new fluid, it appears possible that the pipette, after transferring *a*, would retain some of the bacteria from the *a* drop, and mingle it with the *b* drop, when *b* was changed. Thus possibly the bacterial content of the cultures of the two members of pairs might be on the whole a little more uniform than that of two cultures taken at random; this might cause the rate of reproduction to be a little more alike in the two members of pairs or of split pairs, producing a correlation.

For these and other reasons it was determined to undertake a very extensive experiment, giving numbers sufficiently large to make the results reliable, and at the same time fulfilling all conditions which the experiment demands. For this purpose the two authors of Part II joined forces, since it is physically impossible for one person to care for so many cultures as are required.

We shall call this Experiment 16, in order that there may be no confusion with Experiments 1 to 15 described in the senior author's paper on the Effects of Conjugation (this Journal, vol. 14, 1913, pages 279-391), since in the present paper it is necessary to refer frequently to these fifteen experiments. In Experiment 16 as carried out, 482 strains, derived from the two members (*a* and *b*) of 241 pairs of conjugants, were propagated forty-seven days, an exact record being kept throughout of the number of fissions for each strain; also of the dying out of strains. The important facts as to the culture methods used are as follows:

On March 23, 1912, 250 pairs were isolated from watch glasses that had been taken the night before from a wild culture of *Paramecium caudatum*. Accidents later reduced this number to 241 pairs. As soon as the two members separated, they were transferred to separate slides, giving us thus 482 distinct strains. To each pair a number was given, while the two individuals forming the pair were called *a* and *b*; the progeny of each of these received the same designation. Each strain was therefore designated by a number and a letter, giving precisely its origin and relationship; the designations running from 1 to 241, *a* and *b*.

Until they separated the pairs were kept in the same fluid in which they were found. After separation the individuals were removed to a fluid consisting half of filtered culture water from the original culture from which they came, half of fresh hay infusion. The latter was made by boiling 1 gram of pure timothy hay for ten minutes in 200 cc. of water. The animals were cultivated throughout the experiment in this mixture, which has the great advantage of supplying a uniform bacterial content to all, thus preventing certain strains from developing a peculiar bacterial flora, which would differentiate them from the others.

The two members, *a* and *b*, of a given pair, were placed on separate slides and kept in separate moist chambers. All the *a*'s were kept together in one series, all the *b*'s in another. This effectually prevented the induction of a resemblance between the two by special similarity of conditions, or by transfer of bacteria from one to the other. Of course all the moist chambers were kept near together, under as nearly identical conditions as possible.

The culture fluid was changed every other day, by transferring the animals to two drops of new fluid on a fresh slide. As a rule, one of the authors transferred the *a* series, the other the *b* series, so that there was no opportunity for a correlation to arise by special similarity of treatment of *a* and *b* in certain pairs.

In addition to the slide cultures, it was necessary to keep small stock or reserve cultures, in the mass, of each strain; this gave us 482 such mass cultures to handle, in addition to the 482 slides. These mass cultures were necessary in order to give us accurate knowledge regarding the mortality of the different strains, since the slide culture of a given strain might die out owing to accidental causes, while the mass culture still persisted; this would show that the death was not due to intrinsic causes. No strain is recorded as dead save when both slide culture and mass culture have died out. When the slide culture alone died, it was replaced from the stock culture.

Owing to the irregularities in fission following immediately upon conjugation, it was thought best to make no use of the record of fissions before March 30, one week after conjugation had occurred. The record was of course taken every other day, the exact number

of fissions being recorded. The records were then grouped by ten-day periods, and the number of fissions in these periods were made the units in analyzing the results.

We have thus for each of the 482 strains (so far as they lived through), four records, of the number of fissions for four successive ten-day periods, extending from March 30 to May 8, 1912.

These records are given in table 51, which will be found in the Appendix. This table contains everything that might be distributed in a great number of correlation tables, and at the same time shows much that would not appear in such tables. From it, anyone who so desires can construct tables or repeat the computations on which the results hereafter are based, in order to test their accuracy. Further, we hope that table 51 may serve as a storehouse of material, from which independent study can be made by others on the problems of reproduction and inheritance in these organisms. We shall ourselves make much farther use of it, in papers to appear on inheritance in Protozoa.

Distribution of survivals and deaths among the members of pairs

We may first examine the question as to whether the two members of pairs tend to have the same, or a different, fate, so far as mortality is concerned. Is there a tendency for one to die and the other to live, or are their fortunes alike?

The pertinent data for the four periods of table 51 and for a subsequent period of twenty-one days, are given in table 44. Since the periods of table 51 do not begin till seven days after conjugation, we have in table 44 the data as to the deaths for five periods, of respectively seventeen, twenty-seven, thirty-seven, forty-seven, and sixty-eight days after conjugation. For the first four periods the data are extracted from table 51; for the fifth period of sixty-eight days, the data of table 44 are independent of table 51.

The understanding of table 44, which condenses the results of a vast amount of work, will be facilitated by the following explanation of the various entries.

The total number of strains at the beginning was 482; or for the lower half of the table, where 8 pairs are omitted, 466. Columns (2) and (6)

TABLE 44

Experiment 16. *Paramecium caudatum*. Distribution of deaths among the members of the 241 pairs of table 51, for five periods following conjugation, with the probabilities of the observed results, if the distribution of deaths has no relation to the pairing. The table is divided into two parts: in the upper half are given the data when we include all of the 482 strains that were cultivated ($m = 482$). In the lower half are given the data in case we omit entirely the members of the eight complete pairs that died during the first period, in order to exclude any possibility that their deaths may have been due to the handling of the pairs while still united (see text). This leaves a total of 466 to be considered, in place of 482. See the detailed explanation following the table.

DAYS AFTER CON- JUGATION	DEAD				SURVIVING				PROBA- BILITY OF A DEVIATION SO GREAT AGAINST SO GREAT A DEVIATION AS THAT OBSERVED	
	Individuals	Most prob- able num- ber of pairs	Actual num- ber of pairs	Excess	Individuals	Most prob- able num- ber of pairs	Actual num- ber of pairs	Excess	(10)	(11)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)		
17	55	3	8	5	427	189	194	5	0.00580	191 to 1
27	73	5	12	7	409	173	180	7	0.00242	411.9 to 1
37	79	6	15	9	493	168	177	9	0.000236	4232 to 1
47	92	9	18	9	390	158	167	9	0.000319	3130.9 to 1
68	161	27	40	13	321	107	120	13	0.000343	2915.7 to 1

(Omitting the 8 pairs of the first period, $m = 466$)

27	57	3	4	1	409	179	180	1	0.75815	1 to 3.1
37	63	4	7	3	403	174	177	3	0.15359	5.5 to 1
47	76	6	10	4	390	163	167	4	0.08821	10.3 to 1
68	145	22	32	10	321	110	120	10	0.00402	247.6 to 1

give the number of individuals dead, and alive, respectively, at the end of the periods given in column (1). Columns (3) and (7) give the most probable numbers of complete pairs that would be included among these if there were no relation between pairing and the distribution of deaths; this is determined by formula (1), page 461.

Columns (4) and (8) give, on the other hand, the actual number of complete pairs included; while columns (5) and (9) show how much the actual number exceeds the most probable number. (It is to be observed that this excess is the same for those dead as for the survivors.) Column (10) gives the probability that any deviation so great as this (either by excess or by deficiency) should occur. This is determined in accordance with rule (5), in the Appendix. (If we should compute the probability for so great an excess, it would be much smaller than the probability given in column 10.) This probability shows directly in how many case out of a thousand or a million, et cetera, so great a deviation

would be found as a matter of chance; thus, for the first period, so great an excess would occur but 58 times in 10,000 cases. Column 11, giving the odds against so great a deviation as that actually observed, is obtained in accordance with rule (5).

Attention should be directed first to the upper half of the table only; the lower half is designed to meet a possible difficulty, to be taken up later. Table 44 shows that in this experiment, as in that of Miss Cull, analyzed in Part I of this paper, the number of complete pairs among those dead, as well as among those living, is throughout much greater than would be expected if the distribution of deaths had no relation to the pairing. That is, there is a strong tendency for the fate of the two members of a pair to be alike. The condition found is the reverse of that demanded by the theory of sexual differentiation.

Table 44 shows also, in columns (5) and (9), that the deviation from the most probable number increases as time passes; in other words, there is a tendency in the later periods for deaths to occur among the mates of those that have already died out. This tendency we saw likewise in analyzing Miss Cull's experiment.

The probability of getting the results observed, as a matter of chance distribution, is so excessively small, as shown by columns (10) and (11), that it must be left quite out of consideration. This probability is in fact even much less than that computed for columns (10) and (11), since what we give there is the probability for any deviation of this amount, whether plus or minus. But all the deviations are plus; this decreases the probability greatly.

It is then absolutely clear that there is something in the pairing which tends to cause the two members of a pair to have the same fate.

There is one possible source of error that deserves consideration. In removing the pairs to a slide while still united, it is conceivable that both might be injured, in such a way that both would later die. Thus the deaths of a certain number of the complete pairs might be accounted for.

While there appeared to be no ground for supposing this to be the case in the present experiment, it will be worth while to analyze

the matter in such a way as to exclude even this remote possibility. To do this, we may exclude from consideration all the pairs that died before the end of the first period. If the deaths do not occur within seventeen days of the time the common handling occurred, there is evidently no danger that they were due to this handling; particularly in animals that produce a new generation every twenty-four hours. There were 8 complete pairs that died during these first seventeen days. Excluding these completely, we have 466 lines (233 pairs) instead of 482 lines (241 pairs) to consider. If now we examine the mortality in the later periods among these 466 lines, omitting the 8 pairs that died during the first period, we have the results given in the second half of table 44.

As the table shows, essentially the same relations are shown when we proceed in this manner, as when we include all cases. In every period the number of pairs whose members have the same fate is greater than would be probable if the distribution of deaths had no relation to the pairing. The excess becomes greater and greater in the later periods, showing that there is a tendency for the mates of those dead to die, and of those alive to live. The only difference made by omitting the eight pairs of the first period is to make the numerical expression of the improbability less. Yet even thus it rises to 247.6 against 1, in the last period.

Thus there is absolutely no escape from the conclusion that there is something in the pairing which tends to make the two members of the pairs alike in their fate.

Comparative rate of reproduction in the members of pairs

We may next examine the rate of reproduction in the members of pairs, as shown in table 51, in order to determine whether the members of pairs are more alike or less alike in this matter than would be expected.

The best way to do this is to determine the coefficients of correlation between the members of the pairs, for the diverse periods of the experiment. It may possibly be of interest, however, to first examine directly for some typical period the question

whether the differences between the two members of the pairs is less or greater than that between two strains taken at random. For this purpose we will select the first twenty days of the experiment (sum of the first two periods in table 51). There were 179 pairs (358 lines) that lived through this period. The average difference in the number of fissions between the two members of these 179 pairs, as determined by an examination of table 51, is 2.514 ± 0.122 fissions.

The average difference between strains taken at random is determined in the way set forth on page 424. Mating each of the 358 strains with every other gives us 63,903 pairings; and the average difference turns out to be 3.493 fissions. This is greater than the average difference between the members of pairs by 1.429 fissions, or 56.8 per cent.

Thus again we find that the average difference in fission rate between members of pairs is not greater than that between strains taken at random, as would be required in order to give evidence of sexual differentiation; on the contrary it is very much less. The progeny of members of pairs are more alike than are the progeny of individuals that have not paired.

We may now turn to an examination of this matter by the aid of the coefficient of correlation.

In table 45 are given the coefficients of correlation in number of fissions between the two members, *a* and *b*, of the pairs, for the various periods of table 51. It is important to recall again that the coefficients for the longer periods (20 days, 40 days, etc.) give evidence independent of that for the shorter periods (or at least not bearing any simple and evident relation to the latter).

As table 45 shows, the correlation in rate of fission between the members of pairs does indeed show itself equally when we experiment with very large numbers, and in such a manner as to exclude the possibility that the similarity of the two members is due to environmental similarity. In this experiment on 482 lines of propagation, as set forth in our description of methods, the two members *a* and *b*, of a pair, are kept on separate slides, in separate moist chambers, and handled separately—as a rule, by different persons. All the experimental conditions are such

that any irregularities in the experimental treatment would tend to decrease any correlation that might intrinsically exist. Yet in every period there is a marked coefficient of correlation, which as a rule is ten to twenty times as great as its probable error.

The effect of possible similarity in conditions in the case of strains lying adjacent to each other in the culture chambers was tested in the following way: In the present experiment, where the *a*'s formed one series, the *b*'s another, the two lines 1*a* and 2*a* occupy the two concavities of a single slide; the lines 3*a* and 4*a* the next slide, and so on. Hence the *a* of a given odd numbered pair, and the *a* of the succeeding even numbered pair are throughout immediately adjacent, and therefore under similar conditions. The same relations hold for the *b*'s. If therefore, this similarity of conditions due to adjacence in the experiment has any effect in producing correlation, as suggested on page 434, then this should give a positive coefficient of correlation when we compare the odd numbered line with its following even numbered line.

TABLE 45

Experiment 16. Paramecium caudatum. Coefficients of correlation between the number of fissions for a and that for b (a and b being the two members of a pair), for the various periods embraced in table 51. In each case, save where the word 'all' is appended, the coefficient is based on the fissions of pairs of which both members lived through the period in question. Where the word 'all' is appended, the actual number of fissions for the entire 241 pairs is dealt with, including those that died out before the end of the period in question.

	NO. OF PAIRS	COEFFICIENT OF CORRELATION
First 10 days.....	193	0.3068 \pm 0.0311
First 10 days, all.....	241	0.3770 \pm 0.0264
First 10 days, omitting those that did not divide.....	183	0.3319 \pm 0.0314
Second 10 days.....	179	0.3862 \pm 0.0305
Third 10 days.....	175	0.2690 \pm 0.0334
Third 10 days, omitting those that did not divide.....	174	0.3038 \pm 0.0328
Fourth 10 days.....	167	0.1021 \pm 0.0365
First 20 days.....	179	0.4793 \pm 0.0275
Second 20 days.....	166	0.2094 \pm 0.0354
Total 40 days.....	166	0.3450 \pm 0.0326
Total 40 days, all.....	241	0.3842 \pm 0.0263

We carried out this operation for the second ten-day period (April 8 to 18). There were 346 cases where pairs could be made up in this way, giving a total of 173 pairs. The numbering in the original experiment was not the same as in table 51, since nine of the original 250 pairs were lost by accident; the numbering had therefore to be altered to close up the gaps. Hence the computations were not based on comparing the odd and succeeding even lines in table 51, though the latter would presumably give a similar result.

The coefficient of correlation between the odd numbered line *a* (or *b*), and the even numbered line *a* (or *b*) lying adjacent to it in the moist chambers was, for these 346 lines but 0.0117 ± 0.0363 . That is, there was no correlation whatever. The evidence is therefore strong that this adjacency in position has nothing to do with the production of correlation in the previous experiments, where *a* and *b* were adjacent.

All together, the result of this experiment, with its 482 lines, excludes the possibility that the correlation observed in former experiments was without significance owing to the small numbers employed; or owing to any possible similarity in the environmental conditions of the two members of pairs. The correlation is certainly real, and due to some intrinsic special similarity between the animals that have conjugated.

The data of table 51 show many other relations of extreme interest, bearing on inheritance; on the possible origin of heritable variations; on the fate of the diverse lines of exconjugants; on changes in reproductive power with the lapse of time, and many other points, some of them of great importance for a full interpretation of the significance of conjugation. But in order not to complicate the present paper, which deals mainly with the general question of whether conjugation produces biparental inheritance, it appears best to reserve the analysis of these matters for a later paper on variation and inheritance of these animals. We shall therefore take up here only matters which bear upon the question whether conjugation actually does result in biparental inheritance.

EXPERIMENT 17: 239 SPLIT PAIRS PROPAGATED SEPARATELY FOR
TWENTY DAYS

The only possible explanation for the positive correlation between the members of pairs other than that it is the result of conjugation, is that based on assortative mating, as already set forth on page 432. The two conjugants which united in a single pair are known to be similar in size before union; they might also be similar in other respects, as in fission rate. The coefficient of correlation between the two members might then exist before conjugation, not being due to the latter.

To properly test this matter, it became necessary to therefore carry on an extensive experiment with split pairs, comparable to Experiment 16 with pairs. It would be desirable if the pairs and split pairs could come from the same culture and be propagated side by side. With the large numbers we wished to employ, this was however impracticable.

We therefore collected material from the same ditch from which came the pairs of Experiment 16; put this under the conditions for producing conjugation, and in this way obtained on May 9, 1912, 239 split pairs (pairs of which the individuals were separated before conjugation was consummated). These were propagated in the same manner as were the conjugants of Experiment 16, so that we may refer to page 434 for an account of the methods employed.

Unfortunately the mistake was made in this case of collecting the material from near the mouth of a sewer, where the animals were living in a dense mass, in company with bacteria of putrefaction. Paramecia which come from such conditions do not live well under the conditions necessary for slide cultures, so that the mortality was high. Both members of 190 split pairs however lived through the first ten-day period, and both members of 153 split pairs through a period of twenty days. This gives ample material for making the test we desire.

The data obtained, so far as they bear upon correlation in fission rate between the two members of split pairs, are given in tables 46, 47 and 48. It may be desirable in a later paper on

TABLE 46

Experiment 17. *Paramecium caudatum*, split pairs. Correlation between the two members (a and b) of 190 split pairs, in number of fissions, for the first 10 days (May 9-19) of the experiment. (The more rapid member is designated a, the less rapid b.)

	Fissions of a					
	7	8	9	10	11	12
Fissions of b						
4	1					1
5			1			1
6	2	1				4
7	3	10	7	9	4	33
8		12	24	18	9	63
9			15	27	13	55
10				12	13	28
11					4	5
	6	23	47	66	43	190

TABLE 47

Experiment 17. *Paramecium caudatum*, split pairs. Correlation in number of fissions between the two members (a and b) of 155 split pairs for the second 10 days (May 30-30.)

	Fissions of a							
	9	10	11	12	13	14	15	16
Fissions of b								
4					1			1
5								
6								
7				1				1
8	1	1				1		4
9		1	1	3	1	2		8
10			2	2	7	5		17
11			2	6	14	6	3	32
12				8	14	12	4	59
13					9	11	12	34
14						11	4	16
15							3	3
	1	2	5	20	46	26	26	155

TABLE 48

Experiment 17. Paramecium caudatum, split pairs. Correlation in number of fissions between the two members (a and b) of 155 split pairs, for the entire 20 days (May 9-30) of the experiment.

	Fissions of a											
	17	18	19	20	21	22	23	24	25	26	27	
15		1		1	1							3
16	1				1	2		1				5
17		1	1	2					1			5
18			2	2	4	3	1	1				13
19			2	5	6	6	2	1	3			25
20				2	4	4	3	6	2			21
21					1	10	2	5	6			24
22						6	7	9	5	1		28
23							7	8	4	1		20
24								4	3	2	1	10
25											1	1
	1	2	5	12	17	31	22	35	24	4	2	155

inheritance to publish the complete record, as is done for Experiment 16 in table 51, but the three correlation tables, supplemented by further facts to be given in the text, are sufficient for present purposes.

We shall take (1) the distribution of deaths, and (2) the rate of reproduction, in the split pairs.

Survivals and deaths in the split pairs

Of the 478 lines with which the experiment began, 57 died out during the first ten days, including both members of 8 split pairs. During the second ten days 42 additional lines died out, with both members of 7 additional pairs. Thus during the entire twenty days, 99 lines died out, including both members of 15 split pairs.

Rate of mortality. The data just given show that the mortality in this experiment was considerably higher than in Experiment 16, where we were dealing with pairs that had completed conjugation. In the present experiment, 99 out of 478 members of split pairs, or 20.7 per cent, are dead at the end of twenty days, while

in Experiment 16 but 73 out of 482 members of pairs, or 15.1 per cent, are dead at the end of twenty-seven days (see table 44).

The question will naturally be asked whether this difference has anything to do with the completion of conjugation in the one case, and its lack in the other; whether comparison of the two experiments indicates that the ex-conjugants are more vigorous. To this question the answer *no* must be given; no conclusion whatever can be drawn on this point from comparing the two experiments. The grounds for this are as follows:

1. The matter was directly tested by a later experiment in which both pairs and split pairs came from the same source as the split pairs of Experiment 17, the two sets being subjected throughout to identical treatment. This later experiment, beginning June 11, 1912, was planned for the purpose of comparing the fission rate and mortality of members of pairs and of split pairs for a long period, under identical conditions; as it turned out, it gave data only on the relative mortality of the two. The experiment included 130 pairs (260 lines), and 122 split pairs (244 lines), making in all 504 lines of propagation.

The mortality was much higher than in experiment 17, and *affected pairs and split pairs* to nearly the same extent, though with a slight advantage in favor of the split pairs. On June 17 there remained alive but 34 out of 260 lines descended from pairs, or 13.1 per cent; of the split pairs 48 out of 244, or 19.7 per cent still survived. The experiment was then abandoned, since the survivors were too few to give valuable data on the fission rate.

The difference between Experiments 16, 17, and this later one in respect to mortality was clearly due to differences in the conditions. In Experiment 16 the animals came from relatively pure water, and the experiment was carried on in cool weather; the mortality was low. In experiment 17 and the later one the animals came from extremely foul water (mouth of a sewer), and the experiments were carried on in hot weather; the temperature being still higher in the later experiment (June 11 to 17) than in Experiment 17 (May 9 to 30). The mortality became greater as summer came on; a result in accordance with much

other experience. Hence no conclusions are warranted as to the comparative mortality rate of the descendants of pairs, and of split pairs, from comparing Experiments 16 and 17.

2. This is merely one example of a general principle which is impressed on one throughout all the many experiments on which the present series of papers is based. For comparison as to mortality, vigor, fission rate and the like, one must always compare two lots that are cultivated together, so that all external conditions are the same for each set. Comparisons of such matters in sets cultivated at different times, and therefore necessarily under different conditions, are bound to give fallacious results.

Distribution of survivals and deaths. The experiments which precede the present one show that after conjugation the two members of a given pair tend to have the same fate (either survival or death for both). In the present experiment with split pairs we have the same conditions before us, save that the two paired members have not conjugated. Do such split pairs show the tendency to have the same fate?

In determining this, certain facts are to be noted. It is necessary to use a certain amount of violence in separating some of the split pairs, and this may cause injury. If so, this injury will probably affect both members of the split pair, so that in consequence both may die. Thus one might expect to find among any that die shortly after separation, and without having divided, a greater number of split pairs than would occur if the distribution of deaths were not influenced by this common injury to the two members of the split pairs. It is therefore to the period following the first three or four days of the experiment that we must look for evidence as to any intrinsic relation tending to induce the same fate in the two members. In the case of pairs in which conjugation was completed, we found that the tendency for the two members to have a similar fate increased greatly in the later periods of the experiment.

In the first four days of the present experiment with split pairs there died 26 lines, all save one without fission. Among these 26 lines were both members of 5 split pairs, all dying without fission. Applying our formula (1), we find that when 26

lines die out of 478 the most probable number of pairs is but 1. The actual number is five times as great as the most probable number, so that it is clear that among those dying during these four days something tends to cause both members to die if one dies. Common injury to the two in separating them would have this effect.

Compare this with the relations in the next six days of the first ten-day period. There are left at the beginning of this six days 218 complete pairs (436 lines); of these 29 died during the six days and these included 2 pairs. Thus the number of deaths is greater than in the first four days, but the number of pairs included is less than half as great. By formula (1) we find that when there die 29 out of 436, the most probable number of pairs is 1, so that here we have an excess of 1 pair. The probability for 1 pair is 0.3942; for 2 pairs, 0.1811, so that 2 pairs would occur, as a matter of chance, about half as often as 1. The deviation from the most probable number is 1, and by rule (5) we find that probability of so great a deviation is 0.60582. Hence it is more probable that we should find so great a deviation as this than that we should not, even if the distribution of deaths is quite independent of the mating. There is thus no positive evidence here that the two members of the split pairs resemble each other more than any two individuals taken at random.

In the last sixteen days of the experiment (following the first four-day period), 70 of the lines died out, including 8 pairs. By formula (1) or (2), the most probable number of pairs when 70 lines die, out of 436, is 6, so that we have here a deviation of 2 pairs. By rule (5) we find that the probability of so great a deviation is 0.3038, so that we should find such a deviation in about one case out of three, though the distribution of deaths be quite independent of the mating. Such a result cannot be considered to give any positive evidence that the two members of the split pairs tend to have the same fate.

We may also examine the results for the entire twenty days, comparing it with the results for the first four days. Of the entire 478 lines, 99 died out during the twenty days, including 15 pairs. The probable number of pairs is 10, so that we have an

excess of five pairs over the most probable number. At the end of four days the excess was four pairs, so that in the succeeding sixteen days the excess has increased by but 1 pair; a result that might readily be produced by chance.

Thus in the split pairs it is clear that there is an excess in the number of pairs included among those that die immediately after separation, without fission; and this is what might be expected from the violence sometimes necessary in separating them. But in the remainder of the experiment there is little evidence of a tendency for the two members of the pairs to have a common fate. There is a very slight excess in the number of cases where both members of the split pairs died. If this is not due to chance, it may be the result of the assortative mating which we know to occur.

But when we compare the split pairs with the pairs that had completed conjugation, as in Miss Cull's experiment, and in our experiment 16, we find a very great difference in this respect. In the conjugants, the tendency for the fate of the members of pairs to be alike becomes greater and greater as time passes, until finally we get such extreme results as are found in the third period of Miss Cull's experiment (page 413), or in the later periods of our experiment 16 (table 44, page 437). If in experiment 16 we examine for the conjugants the deaths in the first four days of the experiment, we find but five, including no pairs whatever. It is clear, therefore, that deaths due to common injury of the two members played no part in the case of the conjugants, yet in the experiment as a whole the tendency for the two members to have the same fate is much greater than in the split pairs, where such injury certainly plays a large part.

Summary. The difference between the split pairs and the pairs that have completed conjugation is then in this respect very great, showing that in the conjugants something has occurred to make the two members of the pairs more alike than they were before conjugating. It is clear therefore that *by conjugation the progeny of the two members of pairs are made alike in vitality, so that they tend to have a similar fate, both surviving or both dying out.*

Comparative rate of reproduction in members of the split pairs

The number of fissions for the progeny of the members of the split pairs was recorded for twenty days. We are here interested in the question whether the two members of a split pair tend to be alike in their rate of reproduction, as is the case with the two members of pairs that were not separated before conjugating. For determining this, we have formed tables of correlation for the number of fissions of each member a , as compared with its prospective mate b . The results for the 190 split pairs during the first ten days are given in table 46; during the second ten days (155 split pairs) in table 47; during the entire twenty days (155 split pairs), in table 48. These tables include only the lines in which both members of the split pairs lived throughout the period in question. The coefficient of correlation for such tables is found in accordance with the method set forth on page 429.

For the first ten days the coefficient of correlation for the 190 split pairs (table 46) is 0.1802 ± 0.0335 . The correlation is thus slight, but it may have some significance, since it is about six times its probable error.

For the second ten days (table 47) the coefficient is 0.0438 ± 0.0382 , a result which, based as it is on 155 pairs (310 lines), indicates that there is no correlation between the two members of the split pairs.

Finally, for the entire twenty days (table 48) the coefficient of correlation for the 155 pairs is 0.2620 ± 0.0356 . Here again we have a small positive coefficient which comparison with its probable error indicates to be significant.

There appears thus to be a slight tendency for the prospective members of a given pair to be a little more alike than usual, in rate of fission, even before conjugation has occurred. That is, there appears to be a slight degree of assortative mating with respect to fission rate. This, if it actually exists, is doubtless a secondary result of the assortative mating in size that is known to occur (see Jennings '11). Possibly individuals of similar size tend also to have a similar fission rate.

But this very slight pre-existing correlation, whose existence is even doubtful, since it did not show itself in the second ten days

of the present experiment, is evidently too small to account for the very marked coefficients found when conjugation has actually occurred. While assortative mating may account for a very small degree of similarity between the members of pairs, the process of conjugation itself results in a marked increase of that similarity.

. That is, *the two members of a pair are more alike in their heritable characters after conjugation than before.* As a result, their progeny resemble each other in fission rate more than they would have done if conjugation had not occurred. *Conjugation results in biparental inheritance.*

We shall see the demonstration of this conclusion completed by the results of Experiment 15, to be described in Part III.

PART III

H. S. JENNINGS

CONJUGATION WITHIN A PURE STRAIN: EXPERIMENT 15

The results thus far given require to complete them a study of biparental inheritance in the case of conjugation within a pure strain, where all the conjugants are derived by fission from a single individual. Such an experiment, including both pairs and split pairs, was carried through in August and September, 1912. This experiment has been described in my paper on the Effect of Conjugation (this Journal, 1913, vol. 14, page 343), and the records of the experiment are given in full in tables 34 and 35, in the Appendix of that paper. The results bearing on biparental inheritance, to be given here, are drawn from analysis of the data given in the tables just mentioned. We shall refer to the experiment as Experiment 15, in accordance with the designation used in the former paper, but it was carried through subsequent to Experiments 16 and 17 of the present paper.

Referring the reader then to the paper just cited for a description of the experiment and the detailed record, I shall here mention only essential points. In this experiment 88 lines derived from ex-conjugant members of pairs, and 174 lines derived from

non-conjugant members of split pairs, were cultivated for twenty-four (or twenty-one) days, with extraordinary precautions for keeping the conditions uniform in all the lines. The two members of given pairs or split pairs were kept in separate moist chambers and handled separately.

Unfortunately, for reasons set forth in my former paper ('13), in but a comparatively small number of cases were descendants of both members of pairs or split pairs propagated. Where this was done, however, two lines of propagation were retained from each member. The results are so extremely marked that the number of lines thus obtained turns out to be amply sufficient for solving the problem in which we are here interested.

The question for answer is: Do the progeny of the two members of a pair give evidence of biparental inheritance, in case all the conjugants are originally derived from a single parent? In other words, are the progeny of the two members of the pairs more alike than they would have been if their parents had not conjugated together, but had conjugated with other individuals?

To answer this question for the rate of reproduction, which was the character studied, we must determine (1) whether the progeny of the two members of a pair that have conjugated show any unusual likeness in their rate of reproduction; and (2) whether this degree of likeness occurs also in the members of the split pairs. If we find such an unusual likeness in the members of the pairs, and not in those of the split pairs, this will show it to be the result of conjugation.

An inspection of the 'totals' for the numbers of fissions of the diverse lines during the entire experiment, as given in tables 34 and 35 of my paper on the Effects of Conjugation (this Journal, vol. 14, page 385) will show at once that there is a most striking resemblance in rate of fission between the progeny of the two members of the pair; and that this resemblance is nearly or quite lacking in the case of the members of the split pairs. To bring this out clearly, it will be well to give here a table showing the total numbers of fissions in both cases. The period of time was for the pairs twenty-four days; for the split pairs twenty-one days; from each member (*a* and *b*) of a pair or split pair there was kept

so far as possible two lines of propagation, x and y ; thus we have in the complete case four lines of propagation from each pair. It is in comparing the number of fissions in the lines descended from a of a pair, with those descended from the other member b of the same pair that the striking similarity is seen. The data are given in table 49.

It will be agreed, I think, that the resemblance shown between the two members of pairs is astonishing. Compare for example pair 3, where the number of fissions for a were 34 and 34, for b , 32 and 33, with pair 4, where the numbers are 13 and 12 for a , 12 and 13 for b ; with pair 6, where the numbers are 16 and 16

TABLE 49

Experiment 15. Paramecium caudatum. Numbers of fissions during a period of 24 (21) days, for the progeny of the two members, a and b , of the pairs (conjugation completed), and for the split pairs (conjugation not completed). Each member is represented, when possible, by two independent lines of propagation, x and y .

PAIR	PAIRS				SPLIT PAIR	SPLIT PAIRS			
	a		b			a		b	
	x	y	x	y		x	y	x	y
1	33	37	31	23	1	25	25	23	
2	29	31		30	2	24	25	23	25
3	34	34	32	33	3	18	24	24	22
4	13	12	12	13	4	24	22	25	
5	11		10		5	23	23	24	25
6	16	16		17	6		22	25	27
7	30	32	33	32	7	22		26	
8		29	27		8	21		25	24
9	35	34	32		9		24	25	25
10	29		31	30	10	22	24	26	25
11	32		31	28	11	23	22	24	25
12	33	32	29	25	12	25	23	24	22
13	29		26		13	23	20	21	24
14	32	29	31	30	14	26			24
15	13	10	11	15	15	24	24	23	
16	33		33	27	16	26	24		23
					17	25	23	24	
					18	26		24	
					19	25	23	25	
					20	25			22
					21	26	26	23	21
					22	28	26	26	

for *a*, 17 for *b*; et cetera. When it is recalled that all the lines were propagated with the most extreme precautions for keeping them uniform; that the progeny of *a* and *b* were cultivated in separate dishes, handled separately, and their records separately kept, to be compared only at the end, it will be evident that there is a most striking degree of resemblance, due to intrinsic causes, between the progeny of the two members of pairs.

This comes out strongly when we ask the question whether the differences between the two members of the pairs are greater or less than the differences between two individuals compared at random. This is readily worked out from table 49 by the method described on page 425. The average difference in number of fissions for 24 days between the two members (*a* and *b*) of the pairs (comparing each *a* with each *b* of its own pair) is found to be 2.349 fissions. The average difference when each individual is compared with every other is 8.590 fissions. Thus the average difference between the members of actual pairs is less than one-third as great as that between individuals taken at random. The pairing has increased the resemblance between the progeny of the two members of the pairs in a high degree.

When, on the other hand, we turn to the split pairs we find a complete contrast in these respects. To begin with, we are confronted by the fact, brought out in my previous paper ('13, page 351), that there is no differentiation in rate of fission among the different lines of propagation (or at least extremely little). Thus there is little or no opportunity for any special degree of resemblance between the two members *a* and *b*, of the split pair. But proceeding to examine the matter directly in the second half of table 49, we find indeed that no special similarity between the fissions for the *a* and *b* of split pairs is seen. The contrast in this respect with the conditions found in the pairs is most striking. If we determine the average difference between the two members of the split pairs we find it to be not less than that between individuals taken up at random. Indeed, for the particular case of table 49, it turns out that the difference between the members of split pairs is slightly greater than for random comparisons, the

former being 2.125 fissions, the latter 1.895. The difference is without significance.

To obtain a precise expression of the similarity between *a* and *b* in each case, and to compare accurately the pairs and split pairs in this respect, we must determine for each the coefficient of correlation between the number of fissions in *a* and that in *b*.

The data for this are given in table 49. To determine the correlation between *a* and *b*, we must enter, in the correlation table both representatives (*x* and *y*) of *a* mated with both representatives (*x* and *y*) of *b*, giving us (in the complete case) four entries for each pair (or split pair). In the incomplete cases there will be either one, or two, such entries for each pair. That is, for pair 1 (table 49) we should enter in the correlation table the matings; 33×31 , 33×23 , 37×31 , and 37×23 . Proceeding in this way, we obtain, for the entire period, 43 entries for determining the correlation between *a* and *b* of the conjugants (pairs); 56 such entries for the correlations of *a* and *b* of the split pairs. (In practice it is simpler to compute the correlation by the difference method, without constructing a correlation table, as set forth on page 429, but the above gives the guiding principles, whatever the method used).

The correlation between *a* and *b* was also determined, in this manner, separately for the fissions during the first half of the experiment; during the second half of the experiment, and for the entire period. This gives us three independent determinations, for both pairs and split pairs. The data for these correlations in the partial periods are given in tables 34 and 35 of my preceding paper ('13), so that they need not be repeated here.

The coefficients of correlation between *a* and *b* for the pairs, as compared with the same for the split pairs, are given in table 50.

Table 50 expresses in figures the surprising difference between pairs and split pairs, that is evident on inspecting table 49. Between the two members of the pairs we have for the entire period the extraordinarily high correlation of 0.9238, while between the two members of split pairs there is no correlation whatever (the coefficient being as near to 0 as could be expected).

TABLE 50

Experiment 15. Paramecium caudatum. Coefficients of correlation in numbers of fissions for given periods, between the descendants of the members (a and b) of pairs, and of split pairs,—in the case of conjugation between individuals of the same pure race.

	PAIRS			SPLIT PAIRS		
	Number of days	Number of entries	Correlation between a and b	Number of days	Number of entries	Correlation between a and b
Total time.....	24	86	0.9238 ± 0.0107	21	112	-0.0690 ± 0.0634
First half.....	12	100	0.6325 ± 0.0393	11	120	-0.3175 ± 0.0554
Second half.....	12	104	0.9517 ± 0.0062	10	168	-0.1735 ± 0.0505

The shorter periods are less significant, since fluctuations in the environmental conditions produce in so short a time disturbances affecting the coefficients. Yet they show essentially the same relations as does the entire time; the positive coefficients for the pairs are very high; while for the split pairs there is no positive correlation.

The fact that we find for the first half of the experiment in the case of the split pairs so great a negative coefficient as -0.3175 naturally calls for remark. The other coefficients for the split pairs are not greater than might be expected in the absence of all correlation. But to what is due this negative coefficient of -0.3175 ?

It can, I believe, be affirmed positively that there was nothing in the conditions of the experiment nor in the manner of keeping the records that could give rise to any correlation, negative or positive, between the two members *a* and *b*. They were kept in separate sets, handled separately, and their records were kept separately. With the great number of experiments in progress, it was impossible for the experimenter to have any idea as to whether a correlation was appearing or not; or consciously or unconsciously to manipulate the records in such a way as to tend toward either negative or positive correlation. Moreover the facts to be entered in the record are so entirely clear as to leave no sphere of action for unconscious personal bias. There remain then but two conceivable explanations; the first is that there might be assortative mating of such a character that a slowly reproducing specimen tends to mate with a rapidly reproducing one, giving negative correlation. But aside from the fact that there is no other evidence of this, and that it is almost inconceivable how it could be brought about, (1) it is known that the members of the pure race are not differentiated before conjugation

into slow and rapid lines (see the preceding paper ('13), pages 351-354), so that such assortative mating is impossible; (2) it is not consistent with the result for the entire period in the present experiment, where the correlation for the split pairs is practically 0. We are therefore driven, as the only possible alternative, to the explanation that this negative correlation is merely a chance result, such as would occur now and then if the experiment were repeated many times. Comparison of the coefficient (-0.3175) with the probable error (0.0554) given in the table would seem to raise difficulties for this, but *just what the probable error should be is extremely doubtful*. The probable errors given in the table are based on the number of entries or cases compared, these being given in the first and fourth columns of table 50, for the coefficient under discussion this number is 120. But whether we should use in such cases the number of *pairs* of entries (60 in place of 120), or the number of *actual* lines compared in the experiment (14), or the number of actual pairs (22), for computing the probable error, appears not to be established. If the last named figure is the correct one, the probable error for the coefficient -0.3175 would be ± 0.1293 , which, being more than one third the coefficient, would readily reconcile the latter with the explanation given. Such a change in the method of computing the probable error would not, however, cast any doubt on the validity of the high correlations found for the members of the pairs. For the pairs the probable errors would have to be based on the number 16; this would give for the entire period the coefficient 0.9238 ± 0.0247 ; for the first half; 0.6325 ± 0.1012 ; for the second half, 0.9517 ± 0.0161 —so that the security of the main results is not altered.

Summary. The results of this experiment with a pure strain therefore complete the demonstration, given in former experiments, that conjugation results in bringing about a resemblance between the progeny of the two members of a pair. If any doubt was possible in the case of conjugants derived from wild cultures, there remains none what ever with the results from a pure strain. Here all the prospective conjugants are alike before conjugation; and there is no positive correlation whatever between the progeny of the prospective members of pairs. But after conjugation such correlation comes into existence and rises to an extraordinarily high figure, the progeny of the two members showing a most surprising correspondence in rate of fission.

Question may be raised as to the applicability of the term 'biparental inheritance' to this result. If employed, one of course must not understand by it the production of a resemblance to both parents, for in the present experiment all the parents were

alike in respect to the character studied; yet from some pairs the progeny had a high rate of fission, others a low one. But all recent work has emphasized the fact that the limiting of the concept of inheritance to resemblance is purely artificial; the progeny often inherit from a parent something which makes them quite unlike the parent. In the present case it appears clear that both parents do affect both sets of progeny, otherwise the latter would not be alike; it appears proper therefore to speak of this as biparental inheritance.

To fit the results in this case to any scheme of inheritance known for other organisms appears difficult. This is the first demonstration of biparental inheritance in cases where the two cells that conjugate both continue to exist and reproduce. If the two parents were ordinary heterozygotes, alike in their germinal characters, it does not appear clear why the two sets of progeny should resemble one another so closely. One might expect that they would often receive different combinations of germ plasma. But it is not worth while to speculate on this aspect of the matter till the facts are better known. An experiment with larger numbers of pairs and split pairs, derived from a single individual, would help greatly in the interpretation. But the fact of biparental inheritance in the conjugation of infusoria is clearly established by the present results.

GENERAL SUMMARY

After conjugation in *Paramecium*, usually a considerable number of the lines of progeny descended from the conjugants die out or are weak.

Since in many of these cases the lines descended from the two members of a pair differ in their fate, one dying or reproducing slowly, while the other lives and reproduces vigorously, it has been held that this indicates an incipient sexuality—the 'male' reproducing little or not at all, the 'female' reproducing vigorously. The two members of the pair were thus held to be less alike in their vitality and reproductive power than would be the case if the deaths and the variations in reproductive power were distributed without reference to the pairing.

A method of analysis for determining whether this is correct was worked out, and is described in Part I. Analyzing in this way the data from a large number of experiments performed partly by the present authors, partly by Miss Cull ('07), the following is found:

1. As to survival and death, the fate of the two members of a pair is more alike, not less alike, than would be expected if the distribution of deaths has no relation to the pairing. If one member of a pair survives, the other member tends to survive also; if one dies out, the other tends to die out also. Thus *conjugation has the effect of making the progeny of the two members resemble each other in vitality*.

This effect of conjugation is very decided, so that the number of pairs in which both members survive is much greater than would be the case if the distribution of deaths and survivals were independent of the pairing.

The fact that this likeness in vitality is a consequence of conjugation, and does not exist before it, was shown by an extensive experiment (Experiment 17) with 239 split pairs. The two prospective members, separated before conjugation occurs, show no such strong tendency to likeness in fate as is shown by the two that have conjugated.

2. As to the rate of reproduction, the two members of a pair are more alike, not less alike, in their rate of reproduction, than would be the case if the variations in reproductive vigor are distributed independently of the pairing. Thus *conjugation has the effect of making the progeny of the two members alike in their reproductive power*.

Thus these relations give no evidence for sexuality considered as a tendency for the two members of a pair to be diverse in vitality and reproductive power; the condition actually existing is the reverse one.

What they show is that *biparental inheritance occurs as a result of conjugation*, the vitality and rate of reproduction being affected by both parents, so that the progeny of the two resemble each other in these respects.

Biparental inheritance in rate of reproduction was tested by an extensive study of the coefficient of correlation between the numbers of fissions in the descendants of the two members, for given periods. A decided positive correlation was found, ranging usually at about 0.3 to 0.4, but rising as high as 0.9, in the case of conjugation among the progeny of a pure race.

In the case of mixed cultures it was found that there is a slight and varying degree of correlation due to assortative mating, so that it is shown by the progeny of members of split pairs. Thus *assortative mating occurs with reference to reproductive vigor*; this is probably a secondary consequence of the assortative mating based on size, which was previously known to exist.

But this correlation is increased by conjugation; showing that conjugation produces biparental inheritance.

Demonstration of this is most striking in the case of conjugation among the descendants of a single individual (a 'pure strain'). Here there is no assortative mating with respect to fission rate; all the individuals having before conjugation the same rate. There is thus no correlation in fission rate for the members of split pairs.

But after conjugation there is a differentiation as to fission rate among the pairs, and the two members of a given pair show a most striking correspondence in rate. The coefficient of correlation rises in such cases to 0.9.

Thus biparental inheritance is (for the first time) demonstrated to exist as a result of conjugation in infusoria.

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APPENDIX

FORMULAE USED IN THE ANALYSIS

For convenience of reference there are brought together here the formulae and rules employed in the foregoing paper, for analyzing the question as to the number of pairs included, when a certain number survive (or die), out of a number of paired individuals. In these formulae:

- m = the total number of individuals
 l = the total number of pairs (so that $l = \frac{m}{2}$)
 n = the number of individuals drawn
 k = the number of pairs obtained
 x = the probability for any given number k of pairs

1. To determine the average number (k) of pairs (and for all practical purposes the most probable number of pairs), to be obtained when n individuals are drawn from m individuals forming l pairs:

$$k = \frac{\frac{1}{2} n (n-1)}{m-1} \quad (1)$$

The integral number nearest this average is (with rare exceptions) the most probable number of pairs; where this is not the case, it differs by but 1 from the most probable number.

2. To determine with certainty, for *even* numbers of n , the most probable number (k) of pairs:

$$k = \frac{(n+1) \left(\frac{n}{2} + 1 \right)}{m+3} \quad (2)$$

where the integral portion of the result is the most probable number of pairs.

3. To determine the probability x of any given number of pairs, when n individuals are drawn:

$$x = \frac{n! m - n! l! 2^{n-2k}}{m! k! n - 2 k! l - n + k!} \quad (3)$$

4. Having the probability x_1 for any given number of pairs k_1 , to find the probability x_2 for the next higher number k_2 :

$$x_2 = x_1 \frac{(n - 2 k_2 + 1) (n - 2 k_2 + 2)}{4 k_2 \cdot (1 - n + k_2)} \quad (4)$$

5. To determine how probable it is that there should occur a deviation from the most probable number of pairs as great as that observed:

Determine by (3) and (4) the probabilities for all numbers of pairs deviating less than the given deviation. The sum of these probabilities is the total probability for deviations less than that observed; the difference between their sum and 1 is the total probability of a deviation as high as that observed or given. Dividing the probability for deviations less than that observed, by the probability for deviations as high as that observed gives the odds against a deviation so high as that observed.

6. Stirling's formula for finding $n!$:

$$n! \approx \frac{n^n}{e^n} \sqrt{2 \pi n}$$

where

$$\begin{aligned} e &= 2.7182818 \text{ (or log. } 0.4342944819) \\ \pi &= 3.1415927 \text{ (or log. } 0.4971498726) \\ \sqrt{2\pi} &= 2.506628 \text{ (or log. } 0.3990899342) \end{aligned}$$

TABLE 51

Experiment 16. Paramecium caudatum. Number of fissions for the 482 lines descended from the two members, a and b, of 241 pairs of conjugants, for forty days (March 30 to May 8, 1912). For each line the number of fissions is given for each of four successive periods of ten days each; also the total for the entire forty days. Numbers in parenthesis, as (2), show that the line in question died out during the period indicated, after the number of fissions shown in parenthesis. Totals in parenthesis are for lines that did not live through the entire forty days.

PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS
1	a	8	10	9	8	35	19	a	10	9	7	9	35	37	a	16	9	8	10	37
	b	9	11	9	12	41		b	8	7	8	11	24		b	8	9	10	10	37
2	a	7	11	8	8	34	20	a	8	10	11	12	41	38	a	9	10	10	12	41
	b	6	3	2	2	13		b	10	9	9	10	38		b	9	9	12	13	43
3	a	9	12	5	(7)	(33)	21	a	10	10	8	9	37	39	a	7	9	9	9	34
	b	11	12	9	14	46		b	10	10	8	10	38		b	7	8	9	10	34
4	a	9	11	8	12	40	22	a	11	12	8	11	42	40	a	12	9	11	10	42
	b	9	8	9	10	36		b	11	10	7	9	37		b	10	11	11	14	46
5	a	10	11	9	12	42	23	a	10	6	2	(5)	(23)	41	a	10	11	10	12	43
	b	9	10	8	9	36		b	9	10	9	12	40		b	10	10	12	15	47
6	a	8	12	8	11	39	24	a	12	10	10	12	44	42	a	10	10	9	12	41
	b	10	11	10	13	44		b	11	10	8	13	42		b	8	10	11	16	45
7	a	(1)				(1)	25	a	9	9	9	8	35	43	a	10	10	6	5	31
	b	(0)				(0)		b	7	10	8	8	33		b	10	9	9	13	41
8	a	11	12	9	10	42	26	a	8	9	7	11	35	44	a	10	9	9	13	41
	b	(0)				(0)		b	(0)				(0)		b	11	11	11	13	46
9	a	9	7	8	8	32	27	a	11	12	12	13	48	45	a	9	10	9	15	43
	b	8	6	6	9	29		b	10	9	9	9	37		b	12	9	2	3	26
10	a	11	11	6	4	32	28	a	11	12	9	9	41	46	a	0	(0)			(0)
	b	11	10	9	12	42		b	10	11	11	13	45		b	5	1	1	(0)	(7)
11	a	9	11	10	9	39	29	a	9	8	7	4	28	47	a	5	5	5	4	19
	b	11	9	9	12	41		b	11	11	12	11	45		b	5	7	4	5	21
12	a	9	11	10	(11)	(41)	30	a	11	10	10	13	44	48	a	11	11	9	14	45
	b	9	9	8	11	37		b	11	9	8	12	40		b	10	11	11	14	46
13	a	(0)				(0)	31	a	8	8	7	10	33	49	a	7	(3)			(10)
	b	8	9	7	11	35		b	9	8	8	10	35		b	6	3	(1)		(10)
14	a	9	11	6	3	29	32	a	11	11	9	9	40	50	a	10	9	8	11	38
	b	10	11	8	10	39		b	9	10	8	9	36		b	10	9	8	8	35
15	a	11	6	2	4	23	33	a	(0)				(0)	51	a	11	10	8	12	41
	b	11	10	9	11	41		b	(0)				(0)		b	10	9	10	10	39
16	a	12	12	6	4	34	34	a	7	9	11	13	40	52	a	9	10	7	9	35
	b	9	10	10	12	41		b	7	2	0	(0)	(9)		b	10	11	12	12	45
17	a	(0)				(0)	35	a	10	10	9	13	42	53	a	10	10	9	14	43
	b	10	10	9	11	40		b	9	11	10	10	40		b	12	11	9	13	45
18	a	(0)				(0)	36	a	10	11	8	12	41	54	a	10	10	10	13	43
	b	11	6	5	8	30		b	9	10	10	4	33		b	11	10	10	9	40

TABLE 51 (CONTINUED)

PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS
55	a	10	8	10	10	38	77	a	2	5	3	5	15	99	a	9	8	9	11	37
	b	11	11	9	10	41		b	(6)				(6)		b	9	9	12	14	44
56	a	12	11	11	15	49	78	a	10	12	8	5	35	100	a	10	11	11	14	46
	b	10	9	12	13	44		b	9	10	11	12	42		b	7	9	10	10	36
57	a	11	10	10	14	45	79	a	(0)				(0)	101	a	(0)				(0)
	b	10	11	11	12	44		b	9	9	8	(7)	(33)		b	8	10	10	15	43
58	a	10	9	9	10	38	80	a	11	10	9	15	45	102	a	(0)				(0)
	b	9	10	6	12	37		b	11	10	11	11	43		b	10	12	12	14	48
59	a	9	10	11	13	43	81	a	9	9	8	11	37	103	a	9	5	4	5	23
	b	11	12	11	16	50		b	8	7	6	7	28		b	(0)				(0)
60	a	8	9	10	13	40	82	a	11	11	9	12	43	104	a	9	11	10	11	41
	b	11	11	11	12	45		b	7	5	5	3	20		b	8	10	10	14	42
61	a	13	12	9	14	48	83	a	(3)				(3)	105	a	10	10	9	12	41
	b	9	10	12	13	44		b	10	8	12	12	42		b	11	10	13	14	48
62	a	11	11	11	13	46	84	a	10	9	9	13	41	106	a	12	9	10	8	39
	b	10	10	11	11	42		b	10	8	6	3	27		b	0	(0)			(0)
63	a	12	11	9	11	43	85	a	6	1	3	4	14	107	a	10	11	10	11	42
	b	11	9	11	12	43		b	7	1	4	(0)	(12)		b	(0)				(0)
64	a	10	11	10	14	45	86	a	11	10	11	14	46	108	a	11	11	13	13	48
	b	9	11	10	12	42		b	11	10	7	10	38		b	11	13	12	13	49
65	a	11	13	11	14	49	87	a	9	11	8	13	41	109	a	(0)				(0)
	b	11	9	5	8	33		b	9	9	9	13	40		b	7	9	6	7	29
66	a	11	10	8	13	42	88	a	10	11	9	12	42	110	a	6	(0)			(6)
	b	10	9	8	9	36		b	10	11	8	10	39		b	(0)				(0)
67	a	6	6	5	4	21	89	a	11	11	10	14	46	111	a	11	11	11	13	46
	b	10	9	8	12	39		b	10	10	11	11	42		b	10	10	13	11	44
68	a	11	12	12	15	50	90	a	9	11	10	13	43	112	a	7	6	3	3	19
	b	11	11	10	12	44		b	9	11	12	15	47		b	9	7	12	8	36
69	a	5	6	5	4	20	91	a	10	10	10	13	43	113	a	9	8	8	1	26
	b	11	10	9	10	40		b	10	10	13	17	50		b	8	7	4	11	30
70	a	10	10	9	14	43	92	a	10	10	9	13	42	114	a	(1)				(1)
	b	10	10	9	12	41		b	9	9	11	11	40		b	4	(0)			(4)
71	a	9	8	2	10	29	93	a	11	11	9	13	44	115	a	9	11	10	14	44
	b	4	1	4	5	14		b	10	10	11	12	43		b	8	9	11	12	40
72	a	10	11	12	12	45	94	a	8	6	5	9	28	116	a	8	9	9	11	37
	b	8	10	10	11	39		b	0	(0)			(0)		b	10	10	11	12	43
73	a	14	10	11	14	49	95	a	11	11	10	13	45	117	a	10	10	11	14	45
	b	12	11	10	10	43		b	11	8	12	14	45		b	10	11	13	9	43
74	a	13	10	8	9	40	96	a	11	11	11	13	46	118	a	10	10	11	12	43
	b	11	11	10	12	44		b	10	10	11	12	43		b	9	10	13	13	45
75	a	12	12	11	14	49	97	a	10	7	9	9	35	119	a	8	9	9	12	38
	b	11	9	11	13	45		b	9	9	9	10	37		b	9	8	8	10	35
76	a	10	13	9	12	44	98	a	10	8	6	9	33	120	a	11	11	11	15	48
	b	10	11	13	15	49		b	0	(0)			(0)		b	10	10	9	12	41

TABLE 51 (CONTINUED)

PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS
121	a	10	9	9	13	41	143	a	12	10	9	9	40	165	a	8	10	12	16	46
	b	10	10	11	10	41		b	9	11	7	13	40		b	9	8	9	10	36
122	a	(1)				(1)	144	a	10	10	10	11	41	166	a	8	8	8	13	37
	b	10	10	12	11	43		b	9	10	8	10	37		b	0	(0)			(0)
123	a	10	10	9	(11)	(40)	145	a	10	12	11	14	47	167	a	8	9	9	13	39
	b	9	7	9	11	36		b	9	8	9	13	39		b	(0)				(0)
124	a	10	5	4	(2)	(21)	146	a	8	8	3	(3)	(22)	168	a	9	11	11	15	46
	b	8	7	5	5	25		b	(0)				(0)		b	12	9	9	11	41
125	a	(0)				(0)	147	a	7	5	10	9	31	169	a	9	9	9	15	42
	b	9	9	8	8	34		b	3	1	(0)		(4)		b	6	2	(0)		(8)
126	a	10	10	10	10	40	148	a	9	11	10	13	43	170	a	8	9	9	12	38
	b	9	8	7	6	30		b	9	10	9	11	39		b	7	5	4	3	19
127	a	8	5	3	2	18	149	a	10	10	8	10	38	171	a	1	(0)	*	14	
	b	7	11	10	11	39		b	9	8	7	11	35		b	5	5	5	5	20
128	a	9	10	11	12	42	150	a	(0)				(0)	172	a	8	8	9	10	35
	b	10	10	10	9	39		b	(1)				(1)		b	8	10	9	8	35
129	a	5	5	4	5	19	151	a	10	8	9	11	38	173	a	9	8	8	13	38
	b	5	2	3	6	16		b	9	10	9	14	42		b	9	8	8	8	33
130	a	7	9	11	9	36	152	a	9	7	8	11	35	174	a	5	(0)			(5)
	b	5	2	(0)		(7)		b	9	8	4	11	32		b	10	11	10	13	44
131	a	5	4	4	(1)	(14)	153	a	8	5	7	8	28	175	a	10	9	11	15	45
	b	8	7	8	11	34		b	10	8	8	11	37		b	10	10	10	12	42
132	a	8	9	8	12	37	154	a	9	10	10	13	42	176	a	11	11	11	15	48
	b	8	10	7	10	35		b	9	10	8	14	41		b	(0)				(0)
133	a	6	5	6	(11)	(28)	155	a	(0)				(0)	177	a	11	10	10	15	46
	b	10	12	7	12	41		b	8	9	8	12	37		b	10	5	2	6	23
134	a	10	6	7	7	30	156	a	9	10	12	13	44	178	a	5	9	7	13	34
	b	0	(0)			(0)		b	7	7	6	4	24		b	(0)				(0)
135	a	9	7	10	12	38	157	a	8	11	10	13	42	179	a	(0)				(0)
	b	8	9	8	13	38		b	(0)				(0)		b	(0)				(0)
136	a	4	10	11	14	39	158	a	8	10	10	11	39	180	a	12	6	5	4	27
	b	0	(0)			(0)		b	10	11	10	14	45		b	10	10	9	10	39
137	a	(0)				(0)	159	a	(0)				(0)	181	a	6	7	6	9	28
	b	9	9	7	12	37		b	(2)				(2)		b	(1)				(1)
138	a	11	9	9	13	42	160	a	9	10	12	13	44	182	a	6	9	11	14	40
	b	8	10	9	13	40		b	10	11	8	12	41		b	5	5	4	9	23
139	a	10	10	9	10	39	161	a	(0)				(0)	183	a	9	10	11	15	45
	b	(0)				(0)		b	6	(0)			(6)		b	9	11	11	12	43
140	a	7	9	5	6	27	162	a	7	9	9	8	33	184	a	10	11	9	13	43
	b	(0)				(0)		b	8	8	9	11	36		b	1	9	10	12	32
141	a	9	10	11	11	41	163	a	9	10	9	11	39	185	a	11	11	11	16	49
	b	11	9	8	14	42		b	8	10	11	11	40		b	(3)				(3)
142	a	10	10	9	13	42	164	a	10	11	12	15	48	186	a	9	8	8	12	37
	b	8	11	8	14	41		b	10	10	9	11	40		b	8	9	11	12	40

PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS	PAIR	MEMBER	FIRST 10 DAYS	SECOND 10 DAYS	THIRD 10 DAYS	FOURTH 10 DAYS	TOTAL 40 DAYS
187	a	8	10	10	14	42	206	a	9	3	5	3	20	224	a	11	9	9	11	40
	b	11	9	11	12	43		b	10	10	9	9	38		b	9	11	10	11	41
188	a	11	11	11	15	48	207	a	5	10	10	12	37	225	a	10	12	10	14	46
	b	11	12	12	12	47		b	11	11	11	11	44		b	11	11	9	12	43
189	a	(0)				(0)	208	a	9	8	9	12	38	226	a	2	2	(1)		(5)
	b	7	5	3	4	19		b	9	9	9	10	37		b	(0)				(0)
190	a	10	8	7	10	35	209	a	11	9	9	11	40	227	a	10	10	11	12	43
	b	8	9	9	8	34		b	12	9	7	11	39		b	11	13	10	12	46
191	a	(0)				(0)	210	a	8	7	7	10	32	228	a	10	10	10	12	42
	b	(0)				(0)		b	8	6	8	11	33		b	12	9	9	11	41
192	a	10	10	9	14	43	211	a	10	9	10	12	41	229	a	9	9	10	12	40
	b	9	12	10	12	43		b	10	10	9	12	41		b	10	12	10	11	43
193	a	1	9	8	9	27	212	a	(0)				(0)	230	a	3	(0)			(3)
	b	(0)				(0)		b	2	(0)			(2)		b	10	10	11	12	43
194	a	11	10	8	13	42	213	a	11	9	9	12	41	231	a	10	10	10	12	42
	b	9	13	10	11	43		b	10	8	10	10	38		b	7	5	4	3	19
195	a	11	11	10	11	43	214	a	9	6	7	12	34	232	a	9	9	9	13	40
	b	9	11	9	11	40		b	9	8	9	10	36		b	(0)				(0)
196	a	10	11	8	13	42	215	a	(0)				(0)	233	a	(0)				(0)
	b	3	(0)			(3)		b	1	2	(3)		(6)		b	(0)				(0)
197	a	9	8	8	11	36	216	a	9	3	3	10	25	234	a	10	10	9	14	43
	b	(0)				(0)		b	(1)				(1)		b	11	11	12	12	46
198	a	13	10	10	13	46	217	a	4	8	9	9	30	235	a	6	5	4	5	20
	b	10	12	11	13	46		b	(0)				(0)		b	4	1	2	(1)	(8)
199	a	10	9	7	11	37	218	a	0	(0)			(0)	236	a	(0)				(1)
	b	7	8	9	10	34		b	9	10	9	9	37		b	(1)				(0)
200	a	7	7	6	8	28	219	a	11	10	11	12	44	237	a	8	4	2	4	18
	b	7	5	9	7	28		b	10	12	12	12	46		b	(1)				(1)
201	a	10	7	8	8	33	220	a	9	10	12	13	44	238	a	9	10	10	14	43
	b	9	11																	

THE REACTIONS OF ARTHROPODS TO MONOCHROMATIC LIGHTS OF EQUAL INTENSITIES

ALFRED O. GROSS

FORTY-FIVE FIGURES

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1. INTRODUCTION

Because of the abundance of arthropods, especially insects, the ease with which they may be secured and their adaptability to experimental work they have been used in many investigations of light reactions. A considerable number of these investigations, made both in the field and in the laboratory, have included tests upon the reactions of these animals to lights of different colors. The light used in the field consisted of light reflected from colored objects, such as flowers, colored glass, paper, or cloth. For the experiments performed in the laboratory the different colors were secured by filtering white light through colored solutions or glass, or else from a prism spectrum.

It is very apparent that light reflected from objects in the field, though having the advantage of a nearly natural condition for insects, is very unsatisfactory since the different colors thus produced are not optically pure or of the same intensity. One could not be certain under these conditions whether the response of the organism was due to the intensity of the light or to its specific quality. Furthermore there are so many other uncontrollable factors which may be involved in experiments performed in the field that the results of these investigations are of little value in solving the problems of the reactions of animals to lights of different colors.

Colored lights produced by means of screens of glass or solutions are also unsatisfactory since these lights with the possible exception of the red are never pure but contain more or less of a mixture of rays. Such screens have been found to transmit the invisible heat rays also which when present undoubtedly have their effect in stimulating organisms.

For these reasons the use of colored light reflected from different surfaces or that produced by means of screens is to be avoided in experiments involving a careful analysis of the reactions of animals to this form of stimulus. At present the spectrum is the best means of securing monochromatic lights because of the purity of its colors, but it is little better than light produced

by means of screens, unless the different colors are made equal in intensity.

Many investigators have ignored the important factor of intensity and have ascribed the effect of colored lights on the organism as due solely to the quality of the light. Others have recognized the importance of intensity, but as far as I know the only experiments on animals in which this difficulty has been successfully overcome are those described in two very recent papers published by Day ('11) and Laurens ('11). These two investigators measured accurately the intensity of monochromatic spectral lights by means of an extremely delicate instrument, the radio-micrometer of Boys. This apparatus has opened the way to the correct solution of many interesting problems involving the reactions of animals to colored lights, investigations which heretofore have yielded so many conflicting and perhaps questionable results.

This present investigation was taken up at the suggestion of Prof. G. H. Parker and whatever success has been attained is due to his untiring interest and helpful criticism throughout the whole course of the work. I am also indebted to Dr. H. Laurens for coöperation in the construction of the light generators used in the experiments.

2. HISTORICAL

Much of the earlier work on the reactions of arthropods to colored lights was taken up from a purely psychological point of view. The investigators seem to have worked with the sole purpose of answering the question of whether the lower animals are able to perceive colors as such and, if so, do they perceive the same colors of the spectrum as seen by the normal human eye.

The first recorded experiments, to my knowledge, upon the reactions of the arthropods to colored lights are those of Paul Bert who published an account of his work on *Daphnia* in 1868. He discovered that the *Daphnia* responded to each of the visible colors of the spectrum. When the entire spectrum was thrown

on a trough containing these animals the majority of them collected in the green and yellow, the most luminous region of the spectrum as judged by our eyes. From the results of these experiments Bert concluded that the vision of the lower organisms is the same as it is of the eye of the normal human being. Merejkowsky ('81) experimented with spectral colors upon *Balanus* larvae and the marine copepod *Dias longiremus*. He recognized the importance of the intensity of the light and attempted to eliminate this factor by equalizing the luminosity of the respective colors as judged by his own eye. Under these conditions Merejkowsky found the animals distributed equally in the different colors. He opposes the view of Bert concerning the vision of the lower animals and concluded that the lower crustaceans cannot see the different colors and are conscious of only one color in variations of intensity. "Nous percevons les couleurs comme couleurs, ils ne les perçoivent que comme lumière" ('81, p. 1161).

Lubbock ('79, '81 a) tested the reaction of ants with lights produced by means of colored glass placed over different parts of artificial nests. From his numerous and ingenious experiments he concludes that ants have the power of distinguishing color, that they are very sensitive to the violet and that their perception of color is very different from ours. In two papers published in 1881 and 1883 he describes experiments made with spectral light upon *Daphnia pulex*. His results are in agreement with those obtained by Bert except that he found the *Daphnia* to be responsive to the ultra violet—a fact denied by Bert. In a more recent publication ('04) are described an extensive series of experiments upon bees made in the field. Lubbock placed drops of honey on pieces of colored paper and glass and observed the bees which visited the different colors. The results of his investigations, he thinks, prove that bees can distinguish colors, and that they exhibit a decided 'preference' for the blue. Lubbock's view, that insects can perceive the different colors, is in general agreement with the results of the work by Forel ('88, '01, '04) on ants and bees; the Peckhams ('87, '94) on wasps and

spiders; Perez ('94) and Bethe ('98) on bees; and Fielde ('02) on ants.

Grabner ('83, '84) made an exhaustive series of experiments upon fifty-three different species of animals. Among the arthropods used for these tests twenty-seven were insects and two were spiders. Grabner also attacked the problem from a psychological point of view in an effort to determine whether the lower animals are able to distinguish colors and intensity differences. He studied the distribution of the animals in an apparatus of two compartments illuminated by lights of different colors. Grabner used screens to obtain his colored lights. From the results of his large number of experiments he reached the following general conclusion—"dass die leukophilen oder weissholden Tiere mit geringen Ausnahmen alle blauliebend, die leukophoben oder dunkelholden hingegen rotliebend sind" ('84, p. 245).

Grabner also experimented on blinded cockroaches in an effort to ascertain whether these insects are able to perceive colors and intensities through their chitinous integument. He blinded the cockroaches by covering the surface of their heads with a layer of warm black wax about 3 mm. thick and found when red and blue screens were used the greater numbers still congregated on the side of the apparatus illuminated with red. Grabner concludes from this experiment—"dass die geblendeten Küchenschaben auch farbenempfindlich resp. blauscheu sind" ('84, p. 307).

Gratnap ('83) in a short paper discusses some experiments made, in the open at night, upon the responses of nocturnal Lepidoptera. He placed colored tissue-paper cylinders over kerosene lamps and found the moths exhibited no marked 'preference' for one color over any other. More moths were attracted to the white light than to the colored lights, probably because the light transmitted by the white paper was of greater intensity.

Loeb ('90, '93, '05) studied the reactions of animals to colored lights from the standpoint of the effectiveness of the different rays in orienting the organisms. He objected to the application of the expressions used by the so-called 'anthropomorphists' that animals 'love' or 'prefer' certain colors and 'hate' or 'dislike'

others. Loeb placed caterpillars of *Porthesia chrysorrhoea* under a blue glass and noted that they oriented to the blue light in the same way as when placed in white light. If, instead of the blue glass, he used a red one the larvae remained indifferent. He performed a number of varied experiments on this caterpillar and repeated similar ones on moths of *Sphinx euphorbiae* and *Geometria piniaria*, plant lice, *Musca* larvae, larvae of *Tenebrio molitor* and of the June-bug, *Melolontha vulgaris*, *Limulus polyphemus*, *Polygordius* and several species of copepods. In all these experiments he used only two colors, red and blue, obtained by means of screens. Loeb ('05, p. 182) concluded from the results of his investigations that "The more refrangible rays of the visible spectrum are exclusively or more effective, than the less refrangible rays, in causing the orientation of the animals as is also the case in plants."

Plateau ('97, '99) following the work of Lubbock and Forel, studied the behavior of the bees in an effort to determine whether the insects, in their visits to the flowers, were guided by the different colors. Plateau, instead of using artificial colors, experimented on natural flowers the colors of which, he thought, were of equal brightness. He believed that bees are directed not by color but by the sense of smell, a view in opposition to that held by Lubbock and Forel. Plateau ('97, p. 41) concludes that "Ils (the insects) ne manifestent aucune préférence ou antipathie pour les couleurs diverses que peuvent présenter des fleurs des différentes variétés d'une même espèce ou d'espèces voisines."

The view that bees are not directed to the flowers by the different colors is also held by Bonnier ('79) and Bulman ('99).

Yerkes ('99) made a series of experiments upon the small crustacean *Simocephalus vetulus* with spectral light. When the gas spectrum was thrown on a trough containing these animals, the majority collected in the regions of the yellow and red light. He then placed a prismatic glass box containing an India ink solution between the trough and the spectrum. The greatest depth of the solution was placed over the red and yellow so that the intensity of this end of the spectrum was equal or less than that of the blue and green region. Under these conditions

Simocephalus showed a movement toward the violet. Yerkes ('99, p. 182) conclusions are "*Simocephalus* prefers the orange and yellow of a gas spectrum. This response to the spectrum is a photopathic reaction, and is not, so far as is known, chromopathic."

Hess ('10) has made some recent investigations on the reactions of invertebrates to spectral light. A large part of this work was devoted to experiments upon insects and small crustaceans. Hess placed larvae of *Porthesia chrysorrhoea* at the bottom of a parallel-walled glass vessel. In the dark the larvae remained on the bottom but when the vessel was illuminated from the side by the spectrum, the animals immediately started to crawl upwards in the yellow and green, the most luminous region of the spectrum. In the blue and red the less illuminated areas the larvae remained below. When only two colors, the red and blue of either spectral or screened light, were used, the larvae were most responsive to the blue. This agrees with Loeb's results secured by tests on various animals with these two colors. Hess, however, does not agree with Loeb in believing that the more refrangible rays are the most effective of all the spectrum, since, he has shown that the larvae are more responsive to the green and yellow regions of the spectrum than to the blue end. Hess found that *Hyponomeuta variabilis*, *Dasychira fascelina*, *Lasiocampa potatoria* and *Phragmatobia fuliginosa* responded to the spectrum and to the colors in a way similar to that of the *Porthesia* larvae. Among other arthropods, he tested the effect of colored light on the movements of the eye in *Daphnia* and the aggregation in the spectrum of the crustacean *Cypridopsis*, the larvae of *Culex pipiens*, *Musca* and *Chironomus plumosus*, the adult insects *Coccinella septempunctata*, *Culex pipiens*, bees, house flies and ichneumon flies and the marine crustaceans, *Podopsis slabberi* and *Atylus swammerdamii*. In all of these forms Hess found the yellow-green region of the spectrum most effective in stimulating the animals. The animals which were positive in white light aggregated in the yellow and green; those which are negative, in the violet and red. As to the vision in the invertebrates Hess maintains "dass bei allen bisher untersuchten Wirbel-

losen die Kurven der relativen Reizwerte der verschiedenen homogenen Lichter annähernd oder ganz übereinstimmen mit der Helligkeitskurve für den total farbenblinden Menschen bei jeder Lichtstärke und für den dunkel adaptierten normalen Menschen bei entsprechend lichtschwachem Reizlichte."

In this brief review of the literature pertaining to the reactions of the lower crustaceans and insects to colored lights, we find a great diversity of results and opinions. Merejkowsky ('81) maintains that the difference in response exhibited by the animals to the different regions of the spectrum is due not to the quality of the light but to the relative intensity of the colors. Hess ('10) believes that the relative attractive power for the different colors approach or correspond with the brightness curve of the totally color-blind persons. Gratacap ('83), Plateau ('97, '99), Bulman ('99) and Bonnier ('79) believe that insects are not guided in their movements by color, and Yerkes ('99) states that the factor of intensity has the more important role in the aggregation of *Simocephalus* in the yellow and red regions of the spectrum. On the other hand Bert ('68), Lubbock ('79, '81, '81 a, '83, '04), Graber ('83, '84), Loeb ('90, '93) and others believe that the lower animals perceive or are stimulated by the different colors as such. With the exception of the work on *Simocephalus*, about which there seems to be much difference of opinion, the results of these investigators in general support the conclusion that the blue or the more refrangible rays of the spectrum are most effective in producing a response in the animals which they tested. It is apparent from the work thus far done that it is extremely doubtful just what part the intensity and what part quality of the light has in stimulating the organism. The great variations in results and the conflicting opinions concerning the relative efficiency of the colors is doubtless due to the fact that in none of these experiments was the intensity factor eliminated. Furthermore the lights used in many of the experiments were produced by means of screens which have been shown to be unreliable.

It is the purpose of this paper to present the results of investigations on the reactions of some of the lower animals to spec-

tral lights of equal intensity, measured, not by the human eye, but by accurate physical means. In this work are used five species of insects belonging to three different orders as follows: the larva and the adult of *Calliphora erythrocephala* Meigen and the adult *Drosophila ampelophila* Loew of the Diptera, the *Zeuzera pyrina* Linné larvae and the adult of *Feltia subgothica* Haworth of the Lepidoptera, and the adult *Periplaneta americana* Linné of the Orthoptera.

3. METHODS

The apparatus used in these investigations is the same as that described by Laurens ('11, p. 258) in his paper on the reactions of toads to monochromatic light. An account of the construction of the generators is given in greater detail in a paper published by Day ('11, pp. 310-315). This paper also includes a short description of the radiomicrometer used in measuring and equalizing the intensities of the colored lights.

The essential features of the light apparatus are two light generators placed at opposite ends of a dark chamber 80 cm. deep, 130 cm. long and 70 cm. high. In addition to the dark chamber suitable screens and reflectors were employed to exclude, from the animals during the experiments, any light not proceeding directly from the prism. In order to reduce the amount of diffuse light from the outside to a minimum the entire apparatus was constructed in a large dark room arranged for the purpose. The special accessory apparatus for exposing the animals to the light, which was adapted according to the nature of each organism to be tested, will be briefly described in the account of the experiments given for each species.

The colored lights used in this work were four in number as follows: blue, 420 to 480 $\mu\mu$; green, 490 to 550 $\mu\mu$; orange-yellow, 570 to 620 $\mu\mu$; and red, 630 to 655 $\mu\mu$. These colors were obtained by cutting down the spectrum by means of diaphragms of blackened cardboard with narrow vertical slits of appropriate size. The sources of the lights were Nernst glowers on a 220-volt circuit. In order to equalize the intensity of the four colored lights it was found desirable to use one glower for the red, two

glowers for the yellow, and three glowers for the green and for the blue lights. The finer adjustments of the intensity were accomplished by regulating the size of the diaphragms until each light gave the same reading on the radiomicrometer, that is, the lights were made to contain the same amount of radiant energy. When the lights from each generator were thus equalized any consistent difference in the responses of the animals to the different colors was believed to be due not to the quantity of the light but to its quality.

4. OBSERVATIONS

A. Calliphora erythrocephala Meigen (larva)

Because of the reversal of their phototropism during development, certain species of flies such as the blow-flies are very interesting from the standpoint of their reactions to light. When the blow-fly larva first emerges from the egg, it is either indifferent, only slightly negative, or, as Herms ('11, p. 177) has shown for aggregate larvae, even positively phototrophic to light. As it grows, it becomes more and more responsive to directive light and by the time the feeding period is ended, it is very strongly negative in its response to light. When the adult fly emerges, it is no longer negative but strongly positive. This complete reversal of its phototrophic behavior is closely correlated with the habit of the animal.

1. *Material.* The blow-fly, *Calliphora erythrocephala* Meigen is easily reared in the laboratory throughout the year and hence it is excellent material for experimental purposes. For the investigations about to be described the larvae were kept in culture jars which were constructed by placing large lamp chimneys on earthenware plates of suitable size, and filling them with about two or three inches of moist sand. This sand served to absorb the excess of liquid from the food. Codfish heads were found to be a convenient and desirable food for the larvae. The heads were renewed every few days in order to keep the cultures in the best condition. After the adult flies had deposited their eggs on a piece of fish, the latter was placed in a culture jar

which was left in a warm dark room of comparatively uniform temperature. The larvae selected for experimental purposes were about five or six days' old, an age at which they seemed most active and most responsive to light. The animals were always dark-adapted and were carefully guarded from light for several hours before the experiments.

2. *Methods.* The accessory apparatus for the experiments on the blow-fly larvae was comparatively simple, being merely a small table 18 cm. in height, supporting a thin piece of slate 30 cm. long and 25 cm. wide. This piece of apparatus was placed inside the dark chamber midway between the two generators. The apparatus was arranged so that an animal placed at the center of the slate was illuminated by lights from two opposite sources, each of equal intensity. The surface of the slate was frequently moistened with warm water to facilitate the movements of the larvae and to prevent them from following the old courses of other individuals. In addition to these tests, the larvae were made to trace their own courses on paper with dilute solutions of methylene blue as described by Herms ('11, p: 189). The rate at which the larva moved was indicated by marking on the paper the position of the animals at the end of each ten seconds.

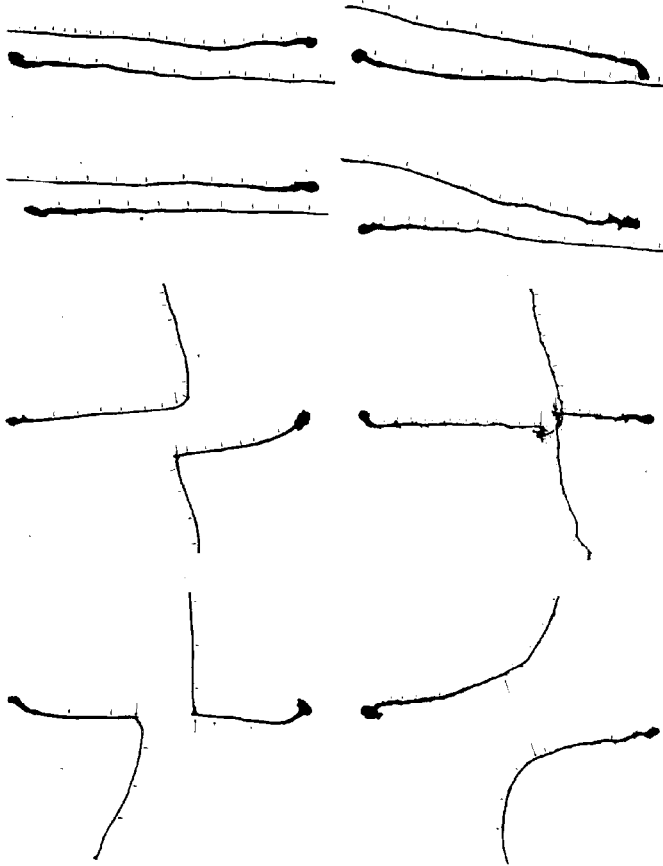
Different individuals, even when of the same age and reared in the same culture under identical conditions, varied more or less in their responsiveness to light. The cause of this difference was not ascertained but probably depended on some physiological condition at present unknown.

3. *Results.* The animals were first tested with each of the four individual colors to determine whether they were responsive to the various wave lengths of light of an intensity used in these experiments. In each test the larva was placed next the side of the slate nearest the source of light. Its subsequent course after orienting was traced on the slate or else on the paper with the methylene blue solution. Each individual was given two tests, the first with the source of light on one side, the second with it on the other. The larva was then put aside and a new

one taken for the next two tests, et cetera. This use of both lights eliminated from the results such errors as might arise from defects in the apparatus, odors, diffuse light, et cetera.

The paths plotted by the larvae under the stimulus of blue, green, yellow and red respectively are shown in figures 1 to 4 inclusive. Under such conditions the larvae oriented and moved away from the light on a course approximately parallel to the direction of the rays. It is apparent from these experiments that the blow-fly larva is responsive to each of the colored lights when of an intensity used in these investigations. As far as I was able to determine from these simple tests, however, the larvae showed no appreciable difference in their response to the several lights.

Opposed lights of equal intensity and of the same spectral quality were then used in testing the larvae. When two lights were used, the larva was allowed first to orient definitely under the influence of one light and after this had been accomplished the other light was thrown on. The position of the larva at the time the second light was turned on was indicated on the record in order to know over what part of the course it was under the influence of the two lights. As the larva changed its position the slate or the record paper, on which it was crawling, was shifted a corresponding amount to keep the animal in the center of the illuminated area and equi-distant from the two sources of light. In general the larvae turned at right angles to the direction of the rays when they were exposed to both lights. Figures 5 to 8 inclusive are records of larvae tested with pairs of blue, green, yellow and red lights respectively. Here again I was unable to perceive any marked and consistent difference in the responses of the larvae to the first three of the above lights. In these cases the angle marking the change in the course was generally sharp and more or less abrupt. When red lights were used the angle in the path made by the larvae was seldom so abrupt but there was more usually a uniform and prolonged curve as shown in figure 8. However, even in red light, if the larva is permitted enough time in which to crawl, it eventually orients so that its median plane comes to lie at right angles to



Figs. 1 to 4 Paths traced by *Calliphora* larvae, in dilute solutions of methylene blue, in response to the single monochromatic lights, blue, green, yellow and red respectively.

Figs. 5 to 8 Paths traced by *Calliphora* larvae in response to balanced pairs of monochromatic lights of equal intensity and of the same spectral quality. Pairs of blue, green, yellow and red lights respectively.

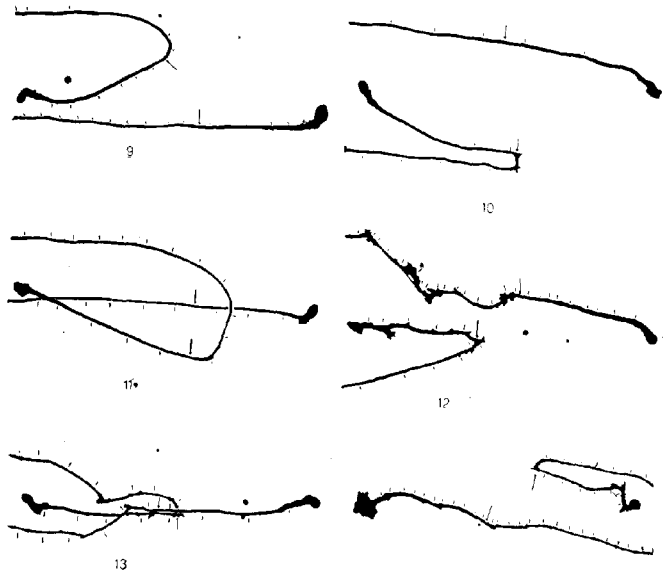
the direction of the rays. This peculiarity in the responses of the animals to red light as contrasted with their reaction to other balanced pairs of colors indicates that red is less effective in orienting the organism than are the other lights, a conclusion borne out by other lines of experimentation.

The foregoing experiments, though showing that the larvae are responsive to all the lights and perhaps least effected by the red, do not show the relative potency of the colored lights. To determine this relation, the four monochromatic lights in all their possible combinations were used in testing the responses of the larvae. Typical examples of the records selected to represent the average of the course taken by the larvae under the respective pairs of lights, are shown in figures 9 to 20. Where red was balanced against blue, green or yellow it is evident from an examination of the records (figs. 9 to 11), that red is least effective in orienting the larvae. A larva started in the red is completely reversed in its direction of crawling when opposed by any one of the other lights. On the other hand a path of the same larva started in the blue, green or yellow is only slightly if at all altered in direction by the red light. Similar results are shown in the records of the reactions under the same pairs but with the lights reversed in direction respectively (figs. 20, 17, and 14).

In pairs where yellow is opposed to the other lights, the red is less effective than the yellow in orienting the animal, but the blue and green are more so (figs. 12 to 14). When the larva is started in the yellow it turns sharply and reverses its course at the point where the blue or green is turned on (figs. 12, 13). In these tests where the larvae were started in blue or green several trial movements were frequently made by a larva when it received the stimulus from the yellow light. The subsequent direction of its course in this case is not reversed but it may be at an angle to the direction of the rays. This experiment shows that yellow when compared with blue and green has an appreciable effect on the larvae although it is much less potent than either of these lights. A larva oriented in the red is reversed in its course by the yellow and one started in the yellow is to

all outward appearances unaffected by the red (fig. 14). These reactions of the larvae to pairs including yellow light demonstrate that yellow is more stimulating to the larvae than red but less than either blue or green.

A study of the records made by the larvae when under the influence of green in opposition to blue, yellow or red (figs. 15 to



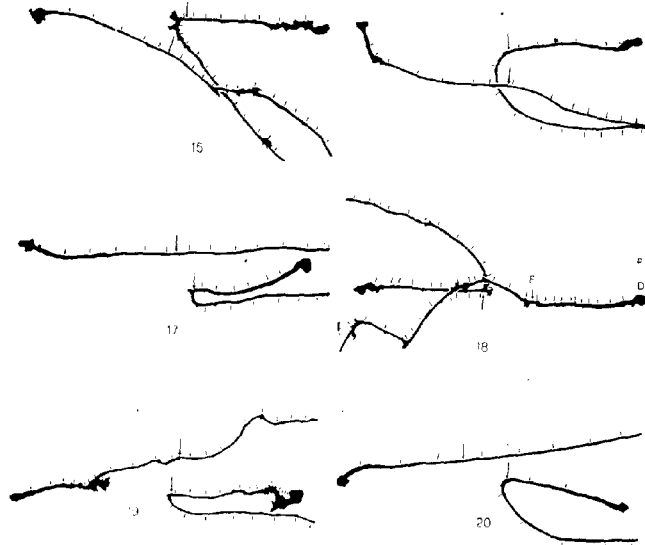
Figs. 9 to 14 Paths traced by *Calliphora* larvae, in dilute solutions of methylene blue, in response to balanced pairs of monochromatic lights of the following pairs:

Fig. 9 Red-blue
Fig. 10 Red-green
Fig. 11 Red-yellow

Fig. 12 Yellow-blue
Fig. 13 Yellow-green
Fig. 14 Yellow-red

17) show that green not excepting blue is the most powerful as an agent for orientation. As might be predicted from the previous experiments the course of a larva started in the yellow or red is reversed when the green is turned on. The course of the same larva oriented in the green is not effected by the red and

only slightly by the yellow. (figs. 16 and 17). When green and blue are opposed, the course of a larva oriented by two lights is at an angle to the direction of the rays as shown in figure 15. If the course of the larva under the stimulus of these two lights had been at right angles to the rays the potencies of green and blue, so far as this experiment is concerned, would have been



Figs 15 to 20 Paths traced by *Calliphora* larvae, in dilute solutions of methylene blue, in response to balanced pairs of monochromatic lights of the following pairs:

Fig. 15 Green-blue

Fig. 18 Blue-green

Fig. 16 Green-yellow

Fig. 19 Blue-yellow

Fig. 17 Green-red

Fig. 20 Blue-red

shown to be equal. The larva, however, regardless of the light in which it was first started was oriented toward the blue in a direction at an angle with the direction of the rays.

In a similar way figures 18 to 20 show that blue is more effective than yellow or red, but less than green. A larva started in the yellow or red is completely reversed in its course by the

blue. In the blue and green pair of lights, the results are similar to those of the green and blue already described. A larva oriented in the blue is turned back at an angle by the green and one started in the green is driven into the blue. The course *D E F* (fig. 18) plotted by a larva in a blue-green pair is interesting since the larva was oriented four different times and each time it took practically the same angle with respect to the direction of the rays. It would be impracticable to reproduce repetitions of the examples used in the foregoing account of the reactions of blow-fly larvae. But since it is desirable to have more than a single average record on which to base an opinion of a result, the records of the respective pairs have been simplified and condensed into diagrams. The construction and use of these diagrams can be explained best by taking an actual example of a reaction record, as for instance, red opposed to blue. In the example (fig. 21, *A*) a larva was placed in a drop of methylene blue at *a* and allowed to creep away from the red light to *b*. When at *b* the blue light was suddenly turned on and as soon as the larva received the effect of the greater stimulus it took the new direction *b e*. In order to classify the records the approximate angle the larva took with reference to the direction of the rays was determined in the following manner. From *b* as a center, where the second light was thrown on, an arc with a radius of an arbitrary length of 8 cm. was drawn intersecting the path, taken by the larva, at *x*. A line was drawn from *b* parallel to the direction of the rays intersecting the arc at *l*. The line *b m* was drawn perpendicular to the line *b l* thus cutting off an arc of 90°. This arc which is now definitely oriented with respect to the direction of the rays was divided into four parts for convenience in combining the records. A circle of convenient size (fig. 21 *C*) was then divided into four quadrants, each of which, as the quadrant in figure 21 *A*, was further divided into four parts and oriented with respect to direction of the rays. The paths traced by the larvae may now be classified on this simple diagram by indicating the position of *x* on the arc of the corresponding sector of the circle. Thus the record of the path *a b e* of the larva in figure 21 *A* is indicated on figure 21 *C* by *x'*.

In the converse of the above experiment, blue opposed to red, the larva was oriented at *c* by the blue light. When at *d* the red light was turned on but the blue being a much greater stimulus than the red, the larva continued apparently on an uninterrupted course to *f* intersecting the arc of the circle at *y*. In a similar way this course may be approximately recorded on the

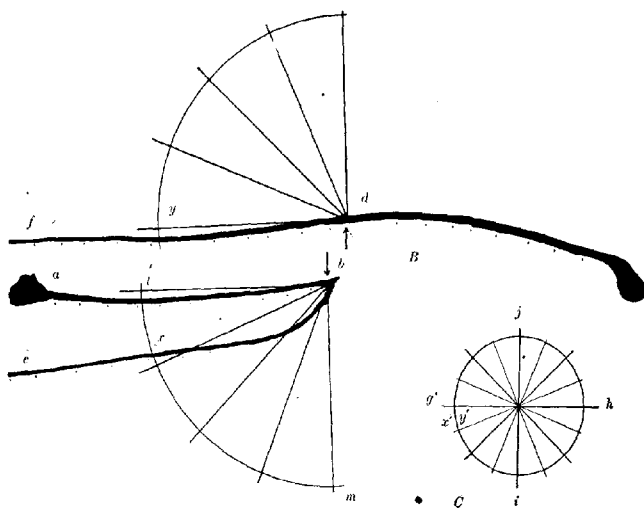
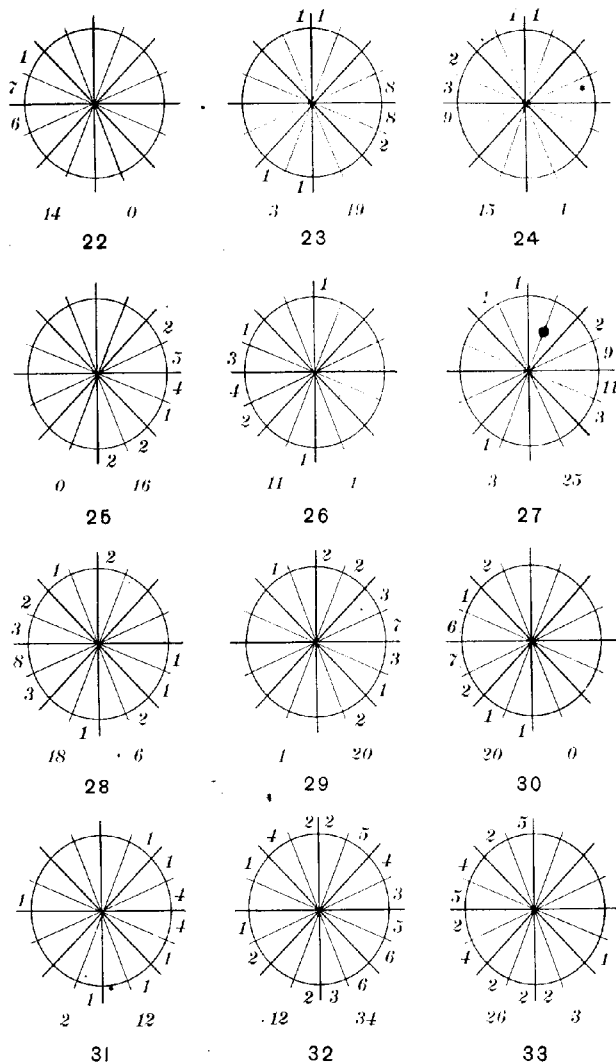


Fig. 21 A typical record of a larva in response to the red-blue pair of lights. A and B are the paths followed by the larva started from the left side and from the right side respectively. The arrows indicate the position of the larva when the second light was turned on. The dots show the position of the larva at ten-second intervals. The quadrants in A and B and figure 21 C are used to illustrate the method of constructing the diagrams of the reaction records of *Calliphora* larvae shown in figures 22 to 33 inclusive.

diagram by *y'* as any number of additional such records may be. A complete diagram of the reaction records of the larvae to the balanced red and blue lights is shown in figure 22. Here out of 14 trials all of the animals were driven by the blue into the red as in the typical record, figure 21 A. The records of a second series of experiments in which the colors were inter-



Figs. 22 to 33 Diagrams illustrating the approximate courses taken by Caliphora larvae in response to the various pairs of monochromatic lights:

- | | | |
|-------------------|---------------------|----------------------|
| Fig. 22 Red-blue | Fig. 26 Red-yellow | Fig. 30 Yellow-green |
| Fig. 23 Blue-red | Fig. 27 Yellow-red | Fig. 31 Green-yellow |
| Fig. 24 Red-green | Fig. 28 Yellow-blue | Fig. 32 Green-blue |
| Fig. 25 Green-red | Fig. 29 Blue-yellow | Fig. 33 Blue-green |

changed are shown in figure 23 and are similar to those shown in figure 21, with the exception that three larvae which were started in the red were not turned in the reverse direction by the blue. They were turned at an angle when exposed to the blue but did not turn completely before creeping off the paper. When such larvae are allowed to continue on another sheet, they usually orient in the course of time in a direction away from the blue light. Such slight irregularities, which may occur in any series, are probably due to corresponding differences in the responsiveness of the organisms. For this reason experiments in which the larva is started first in one light and then in the other are highly important in serving as an effective check throughout these records. In each diagram (figs. 22-33), though the records are plotted all on one side, they represent records one-half of which are from larvae started on the right side and the other half on the left side in the respective colors of each pair. A greater number of observations were made for the green and blue pairs than for any other, since the relative differences of the stimuli produced by these lights, is apparently less than that in the lights of the other pairs.

These combined records of the blow-fly larvae reactions substantiate the results previously shown in the reproduction of the paths plotted by the larvae, namely, green is the most effective and the red the least so, of the four monochromatic lights, in orienting the larvae. The blue and the yellow are intermediate between these extremes but the blue light is much stronger in its effect than the yellow. The effectiveness of the four colors, therefore, is in the order, beginning with the strongest; green, blue, yellow, red.

B. Calliphora erythrocephala Meigen (adult).

1. *Material.* The adult blow-flies used in these experiments were reared in the laboratory from the cultures of larvae used in the foregoing experiments. The flies were easily kept alive and in good condition by feeding them on sugared water. They were placed in a large screened cage provided with a device for

removing them to small glass jars as required. In order to dark-adapt the flies, the jars containing two to five individuals each were placed in the dark at least an hour before using them in the experiments.

2. *Methods.* The essential part of the apparatus used in these tests was an elongated glass cylinder or tube 6 cm. in diameter and 40 cm. long supported on a base 14 cm. high. The base was a black box, 16 cm. wide and 20 cm. long, open on one side to allow the experimenter to place or remove the glass jars containing the flies. This apparatus was placed midway between the generators in such a way that the center of the cylinder was directly in the center of the field of light and its axis parallel to the direction of the rays. A funnel opened into the cylinder from the dark chamber of the box below. Since the flies are strongly negatively geotropic, they readily crawl upwards when freed in the funnel. As they leave the narrow opening leading into the illuminated region they are oriented to one side or the other depending on which light exerts the greater stimulus. Mechanical counters were used for recording the number of flies as they left either end of the apparatus. The flies were not used a second time but a fresh lot of dark-adapted individuals was taken for each new set of records.

3. *Results.* The flies were tested first with single lights to determine whether they were responsive to each of the four colors of an intensity used in these experiments. In all of these tests the direction of light rays was interchanged for each set of individuals, but for a matter of convenience all the records of any one color are combined. When the single lights are thus compared with darkness the flies were found to be distributed as shown in table 1. The records of the experiments shown in table 1 demonstrate the strong positive phototropism of the blow-fly to each of the four monochromatic lights. A greater number of flies were negative or indifferent to the red or yellow than there were to the blue or green, a result which indicates that the more refrangible rays are more effective than those at the opposite end of the spectrum. This relative efficiency of

TABLE 1

Reactions of Calliphora adult to single monochromatic lights

COLORS	NUMBER OF REACTIONS		PERCENTAGE OF POSITIVE REACTIONS
	Positive	Negative	
blue.....	113	1	99
green.....	110	5	96
yellow.....	150	9	94
red.....	106	26	80

the colors is corroborated by the results of further experiments, to be described later, on the adult blow-flies with balanced lights of different colors.

Flies were next tested in the cylinder illuminated by paired lights of the same color and same intensity. About 100 flies used in each of four such pairs were found to be distributed to the two sides as shown in table 2. Theoretically the distribution ratio of the responses of the flies to balanced lights of the same colors should be 50 % : 50 %, but when comparatively small numbers are used slight deviations, as in the above results, may be expected. The percentages, however, even with these limited number of trials, vary only from one to three units from the expected ratio and show the degree of reliability one may place on the records of the reactions of the adult flies to balanced lights. With each new set of trials with any pair of colors the direction of the rays was reversed to eliminate from the results any errors which might arise from defects in the apparatus.

TABLE 2

Reactions of Calliphora adult to pairs of monochromatic lights of the same quality and of equal intensity

COLORS		NUMBER OF REACTIONS		PERCENTAGE OF REACTIONS TO THE LEFT
Left	Right	Left	Right	
blue.....	blue	47	53	47
green.....	green	55	49	51
yellow.....	yellow	65	61	51
red.....	red	62	69	48

The records of the responses when the flies were subjected to the respective combinations of different colors are given in table 3. For convenience in comparing the relative potencies of the colors, as expressed in per cents of reactions, table 4 is introduced, in which each color is readily compared with every other color and with darkness.

In table 4 the colors heading the columns are compared with those facing the lines. For example, if we wish to know what percentage of the flies went to the green in the green-yellow pair of lights we look for the number in the column headed green and in the line opposite the yellow. The number 79 indicates that in the experiment with this pair of lights 79 per cent of the individuals tested went to the green away from the yellow.

TABLE 3

Reactions of Calliphora adult to pairs of monochromatic lights of equal intensity

PAIRS OF MONOCHROMATIC LIGHTS		NUMBERS OF FLIES GOING TO EACH LIGHT		TOTAL NUMBERS OF FLIES TO EACH LIGHT		PERCENTAGE OF FLIES TO MORE REFRACTIVE LIGHT
Right	Left	Right	Left	More refractive	Less refractive	
blue	green	92	50	171	100	63
green	blue	50	79			
blue	yellow	55	22	106	40	72
yellow	blue	18	51			
blue	red	33	6	113	14	89
red	blue	8	80			
green	yellow	70	35	167	43	79
yellow	green	8	97			
green	red	93	18	167	23	87
red	green	5	74			
yellow	red	66	22	129	35	79
red	yellow	13	63			

TABLE 4

Combined records of Calliphora adult reactions

COLORS	BLUE	GREEN	YELLOW	RED	DARKNESS
blue.....	47	37	28	11	1
green.....	63	51	21	13	4
yellow.....	72	79	51	21	6
red.....	89	87	79	48	20*
darkness.....	99	96	94	80	

In the reverse of this pair, yellow-green, the number 21 in the column headed by yellow and in the line faced by green indicates that 21 per cent of the flies used went to the yellow. In a like manner the results obtained with any pair of lights may be readily ascertained.

From the above series of records it is at once apparent that the adult blow-flies are responsive to all the colors, and, of the lights used in these experiments, are affected most by those containing the more refrangible rays. The effectiveness of the colors in stimulating *Calliphora*, therefore, are in order, beginning with the strongest: blue, green, yellow, red.

C. Drosophila ampelophila Loew (adult)

The pomace or little fruit fly, *Drosophila ampelophila* Loew, is a very common insect for experimental work, not on light alone, but in a great diversity of lines of investigation, because of the ease with which vast numbers can be reared and handled in the laboratory. Carpenter ('05) has demonstrated the strong positive reaction of *Drosophila* in its response to white light, but no one, as far as I am able to discover, has made careful investigations of the reactions of these insects to monochromatic light. The tests made with *Drosophila* in the following investigations were carried out in practically the same way as those made with the adult blow-fly. Since the pomace fly is so closely related to the adult blow-fly and has the same type of visual mechanism as that insect, one would naturally expect in experiments with it to obtain results similar to those from the blow-fly.

1. *Material.* A continuous culture of *Drosophila* derived from an original stock secured during the summer was maintained in the laboratory throughout the winter. They were reared in large glass jars which contained a supply of decaying bananas on which the insects fed and deposited their eggs. By inverting a large glass funnel over such a culture and directing it upwards and towards a strong light the flies can, because of their strong reactions, be easily conducted through the funnel into small jars.

About 15 to 20 individuals were thus transferred to each of about 25 small 4-ounce wide-mouth jars for each of the series of tests. The flies were not reared in the dark room but were always dark-adapted before they were used in any of the light experiments and, as in the case of the adult blow-flies, they were never used a second time immediately after having been exposed to the light of the apparatus.

2. *Methods.* The apparatus used for the *Drosophila* was essentially the same in principle as that used for the blow-flies and differed only in some minor details. The tube leading from the funnel in this apparatus was smaller and its opening into the illuminated cylinder was partially obstructed by two pieces of cork which prevented the flies from going upwards too rapidly. There was also a device to enable the experimenter to close completely the aperture when so desired.

TABLE 5
Reactions of Drosophila to single monochromatic lights

COLORS	NUMBER OF REACTIONS		PERCENTAGE OF POSITIVE REACTIONS
	Positive	Negative	
blue.....	191	10	95
green.....	183	42	81
yellow.....	160	55	74
red.....	152	60	71

3. *Results.* To determine whether all the lights used were effective in stimulating the animals the flies were permitted to enter the cylinder illuminated by a single monochromatic light from one end only. In each of these tests about 200 individuals were used, which were found to react as represented in table 5. This experiment demonstrates the strong directive effect that each of the colored lights used in these tests have on *Drosophila*, and gives further evidence of the strong positive phototropism of these insects. A greater percentage of the individuals were negative when tested with the yellow or red lights than when the blue and green were used. This result suggests the fact subsequently established that the lights having the more refrangible rays exert the greatest directive stimulus on the pomace flies.

The flies were then tested with balanced lights of the same colors to determine to what extent the percentages of the responses can be depended on to express the relative potencies of the lights used. The results of this test are shown in table 6.

The extreme amount of deviation in the percentages in table 6, which is only 4 units less than the expected theoretical ratio of 50 % : 50 % is found in the red.

TABLE 6
Reactions of Drosophila to pairs of monochromatic lights of the same quality and of equal intensity

COLORS		NUMBER OF REACTIONS		PERCENTAGE OF REACTIONS TO THE LEFT
Left	Right	Left	Right	
blue.....	blue	82	86	49
green.....	green	41	40	50
yellow.....	yellow	46	43	52
red.....	red	53	62	46

In the following experiments on the responses of *Drosophila* to balanced lights of different colors not less than 300 and sometimes as many as 500 individuals were used in each combination of colors. The large numbers used tend to reduce the size of the error of the results and to give a more correct representation of the relative efficiencies of the four colored lights. Table 7 is a summary of the reactions of the pomace flies to balanced pairs of monochromatic lights of different spectral qualities.

A number of tests were made in which the flies were caught in the retaining cylinder and run through a second series of trials. This was done to determine whether or not flies exhibiting a negative reaction to the individual colors or to the more refrangible color of any pair of lights are permanently negative or indifferent. The factor of mechanical stimulation of the flies was eliminated in these experiments by allowing the flies to rest and to become dark-adapted before using them in a second test. The results of five such tests are shown in table 8.

The results shown in tables 5, 6 and 7 are combined and condensed into the simple table 9.

TABLE 7

Reactions of Drosophila to pairs of monochromatic lights of equal intensity

PAIRS OF MONOCHROMATIC LIGHTS		NUMBERS OF FLIES GOING TO EACH LIGHT		TOTAL NUMBERS OF FLIES TO EACH LIGHT		PERCENTAGE OF FLIES TO MORE REFRACTIVE LIGHT
Left	Right	Left	Right	More refractive	Less refractive	
blue	green	259	79	502	128	79
green	blue	49	243			
blue	yellow	162	35	268	39	87
yellow	blue	4	106			
blue	red	206	17	423	31	93
red	blue	14	217			
green	yellow	124	33	323	102	76
yellow	green	69	199			
green	red	152	42	391	115	77
red	green	73	239			
yellow	red	174	69	366	174	68
red	yellow	105	192			

TABLE 8

Records of tests of the re-distribution of Drosophila adults

COLORS		NUMBERS OF FLIES GOING TO EACH LIGHT IN FIRST TEST		NUMBERS OF FLIES CAUGHT FOR SECOND TEST		DISTRIBUTION OF FLIES IN SECOND TEST	
Left	Right	Left	Right	Left	Right	Left	Right
blue	red	67	9		6	5	1
green	red	42	13		8	5	3
red	yellow	24	34	11		4	7
red	green	20	67		7	1	6

TABLE 9

Combined records of Drosophila reactions

COLORS	BLUE	GREEN	YELLOW	RED	DARKNESS
blue.....	49	21	13	7	5
green.....	79	50	24	23	19
yellow.....	87	76	52	32	26
red.....	93	77	68	46	29
darkness.....	95	81	74	71	.

The colors indicated on the lines are compared with those of the columns as was done in the table of percentages for the adult blow-fly. For example in the blue-red pair the number 93 in the blue column opposite the red denotes that in this test 93 per cent of the flies went to the blue and away from the red. In a similar manner the result of the experiment with any pair of lights may be readily determined.

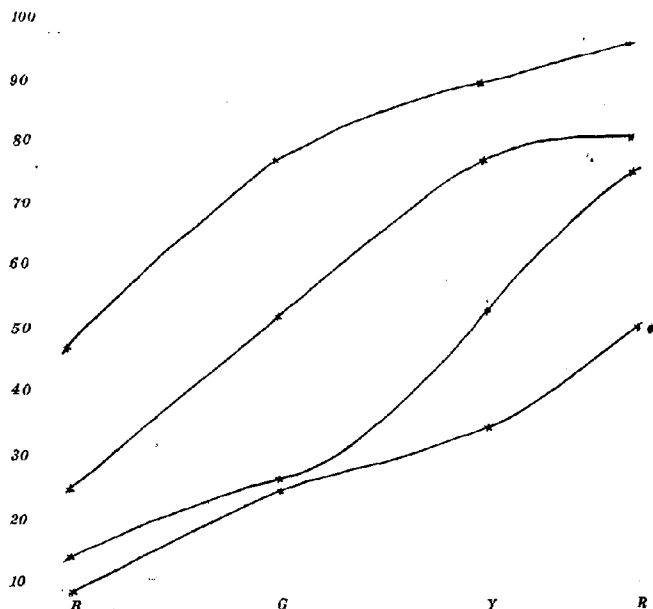


Fig. 34 Curves representing the percentages of the responses of the pomace flies to the four monochromatic lights. Percentages of responses of animals to the respective colors as ordinates and wave lengths as abscissae. The wave lengths of the middle band of each light are indicated by points marked on the axis of the abscissa.

The results presented on the foregoing pages may be expressed graphically in a series of curves in which each color is compared with each of the other four colors (fig. 34).

The results of these experiments demonstrate that of the four colors the ones containing the more refrangible rays exert the

greatest directive stimulus on *Drosophila*. The effectiveness of the colors beginning with the strongest is, therefore, in the following order: blue, green, yellow, red.

D. Zeuzera pyrina Linné (larva)

The lepidopterous insects are, as far as I know, unlike the dipterons in that there is no reversal of their phototropism during development. Both the caterpillars and the adults of the species of moths and butterflies¹ known to be responsive to directive light are distinctly positively phototropic.

1. *Material*. The larva of the European wood leopard-moth, *Zeuzera pyrina* Linné, used in these experiments, was accidentally introduced into this country from Europe, and has since proven to be a serious pest to our shade trees and shubbery. This species of moth has not, to my knowledge, ever been the subject of light experiments, but Chapman ('11, p. 15) states: "The larvae when they first hatch exhibit a marked positive phototaxis for they make their way to the tips of small branches to the axis of leaves or to nodes and buds near the tips, and at once bore into the woody tissue."

This suggests an interesting correlation of the positive phototropism of these insects with their habits during the early stage of their life history.

Unlike the blow-fly larvae the leopard-moth larvae are most responsive during the early part of their free life. They become more and more sluggish in their reactions to light as they approach the pupal stage which, according to Chapman, is not attained until the second year.

The larvae were abundant in the twigs and branches of many of the trees and shrubs, especially the lilacs in the vicinity of the Museum of Comparative Zoölogy, Cambridge. The lilacs provided an abundant and convenient source of material. A considerable number of the larvae used in these experiments were secured by Mr. Chapman from the trees and shrubbery of Boston Common. Since the larvae are somewhat troublesome

¹The reactions of *Venessa antiopa* Linn. is an apparent exception. These butterflies when alighted turn away from bright sunlight. Parker ('03).

to keep in the laboratory in a good vigorous condition new specimens were secured each time it was desired to make a series of tests. Parts of branches were taken into the dark room from which the contained larvae were not removed until they were required in the experiments. These investigations were made during October and November, a time too late for specimens of newly hatched larvae but all the individuals of the first winter exhibited a very strong positive phototropism.

2. *Methods.* The apparatus used for these experiments on the leopard-moth larvae was essentially the same as that employed for the larvae of the blow-fly. The paths followed by the larvae were, however, traced with a pencil, since methylene blue could not be used to an advantage because of the waxy nature of the larval integument. The pencil tracings were made by following the course of the animal in such a way as not to interfere with its movements or with the rays of light impinging on them. The larvae when cold did not respond very readily to the light, hence it was frequently found desirable to warm the slate, by means of an electric heater placed beneath the apparatus, to a uniform optimum temperature.

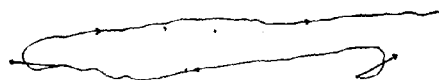
3. *Results.* The accurate responsiveness of the larvae to the different colors is demonstrated best in experiments with single colors in which the direction of the light is suddenly reversed. A typical record of the result of such an experiment, in which a larva was tested with blue light, is illustrated by figure 35. On this test the larva was placed at *A*, in the position indicated by the arrow, in the midst of a beam of light coming from the left. The larva immediately oriented and was permitted to crawl towards the light undisturbed, to the position at *B*. At this point the direction of the light was suddenly changed, whereupon the larva immediately reversed its course in a direction towards the new source of light at the right. The similar results of many such tests made with each of the colors demonstrate the positive phototropism and the marked responsiveness of the leopard-moth larvae to lights of an intensity and quality used in these experiments. The relative efficiency of the four lights was obviously not determinable from the results of these simple

preliminary tests with single colored lights. However, when the larvae were tested with red, they seemed to orient less accurately than when stimulated by any one of the other colors, a result indicating that the less refrangible rays are least effective.

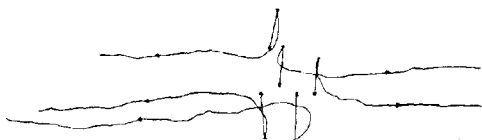
In the experiments with balanced pairs of monochromatic lights the larvae were started in the middle of the slate or sheet of paper and in the center of the field of light. They were placed with their axes at right angles to the rays of light. This position gave the anterior end of the animal, which, presumably, is the only part sensitive to light, equal opportunity to stimulation by the lights of equal intensities coming from opposite sources.

When the larvae were exposed to paired lights of the same colors, they oriented, not with their axes perpendicular to the light rays, as was the case with the blow-fly larvae, but toward one light or the other approximately parallel to the direction of the rays (figs. 36 and 37). When paired lights of different colors were used the larvae turned toward the light containing the more refrangible rays (figs. 38-43).

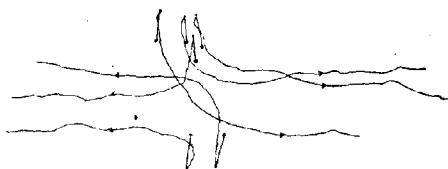
In addition to the method used in the experiments just described, the larvae were started at one edge of the slate and allowed to orient definitely to the rays of one light before turning on the opposing light. Figures 44 and 45 are records of the paths taken by a larva when thus tested with the green-yellow pair of lights. In the records reproduced in figure 44, the larva was started at *A* and was allowed to crawl towards the yellow light to the position at *B*, when the green light from the opposite was turned on. When thus exposed to the influence of two lights the larva did not, in this case, reverse its course immediately but continued to *O* before orienting to the green. The distance crawled by the larvae after the green light was turned on varied considerably in the several tests with the same and different larvae. In some cases the response was immediate and in a very few tests the larvae crawled off the sheet of paper towards the yellow light without exhibiting the least evidence of the effect of the green. The visual organs, the larval eyes or ommata, on the anterior end of the larvae are not in a favor-



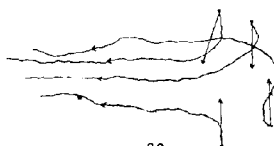
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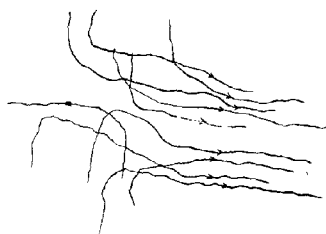
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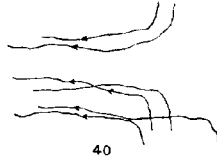


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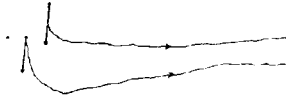
Figs. 35 to 45 Paths followed by *Zeuzera* larvae in response to monochromatic lights of equal intensity, as described on pages 496 to 500 inclusive. The approximate length of the larvae as well as their position and direction of travel is indicated by the arrows. Action of lights successive in figures 35, 44 A and 45 A; in all other cases simultaneous.

Fig. 35 A Blue alone at left
Fig. 35 B Blue alone at right
Fig. 36 Blue-blue

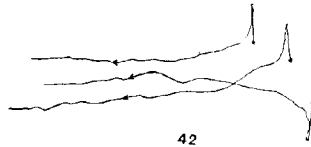
Fig. 37 Red-red
Fig. 38 Blue-green
Fig. 39 Green-blue



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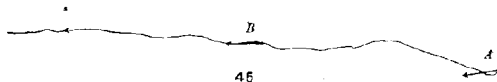
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43



44



45

Fig. 40 Green-yellow

Fig. 41 Yellow-green

Fig. 42 Yellow-red

Fig. 43 Red-yellow

Fig. 44 A Yellow alone at right

Fig. 44 B Green-yellow

Fig. 45 A Green alone at left

Fig. 45 B Green-yellow

able place to be stimulated by any color when the body is oriented away from its source, hence in the above experiment, the variation in time of response probably depends, to a certain extent, on the accidental exposure of the ommata to the green light.

In the record shown in figure 45 the larva was started from the right at *A* in the green light and was allowed to crawl to *B* before the yellow light from the opposite side was turned on. In no case of the many tests made was the larva reversed in its course by the yellow when the latter was opposed with green. In similar experiments made with each of the remaining pairs of lights the larvae were shown to be most responsive to the colors having the more refrangible rays. The effectiveness of the colors in stimulating the leopard-moth larvae corresponds to their sequence in the spectrum, namely, beginning with the strongest: blue, green, yellow, red.

E. Feltia subgothica Haworth (adult)

1. *Material.* Adult specimens of the leopard-moth could not be obtained at the time of these experiments, but I was fortunate in securing numbers of another species of Lepidoptera (*Feltia subgothica* Haworth). These moths were very common around the arc-lights at night during the month of September. Through the kindness of the Bussey Institution at Forest Hills an excellent arc-light insect trap was placed at my disposal by means of which the necessary material for the following experiments was secured. The moths thus collected were placed in ventilated pasteboard boxes, which were shielded from the light until the specimens were required in the experiments. In addition to this precaution, since it was desirable to have each individual dark-adapted, none of the moths were used in two succeeding experiments.

2. *Methods.* The chief feature of the apparatus was an elongated glass chamber, 10 x 10 x 45 cm., which had an opening at the middle of the side nearest the experimenter through which the moths could be liberated from the paper boxes. This apparatus, as that of the previous experiments, was placed in the middle of the field of light midway between the two generators.

A moth allowed to enter the chamber was free to go to one side or the other, but since it is positively phototropic it would be expected, other factors being equal, to go to the light exerting the greater stimulus.

3. *Results.* The moths reacted to the various pairs of lights as shown in table 10. The numbers represented in table 10, though too small to warrant a comparison of the percentages of the responses to the various colors, nevertheless show that of the four colors used those of the more refrangible rays are more effective in stimulating and orienting the adult *Feltia subgothica*. The effectiveness of the four colors, therefore, is in the order beginning with the strongest: blue, green, yellow, red.

F. Periplaneta americana Linné (adult)

The adult cockroach, *Periplaneta americana*, has, so far as I know, never been used in experiments with spectral lights. Graber ('84) has, however, made a series of investigations with a closely allied form, *Blatta germanica* Linné, with colored lights produced by means of screens.

TABLE 10
Reactions of Feltia to pairs of monochromatic lights of equal intensity

PAIRS OF MONOCHROMATIC LIGHTS		NUMBER OF MOTHS GOING TO EACH LIGHT		TOTAL NUMBER OF MOTHS TO EACH LIGHT	
Left	Right	Left	Right	More refractive	Less refractive
blue	blue	20	18		
green	green	7	9		
yellow	yellow	6	9		
red	red	10	13		
blue	green	12	6		
green	blue	1	11	23	7
blue	yellow	13	3		
yellow	blue	7	18	31	10
blue	red	11	3		
red	blue	6	23	31	9
green	yellow	12	0	22	5
yellow	green	5	10		
green	red	14	3		
red	green	4	19	33	7
yellow	red	16	9		
red	yellow	7	17	33	16

1. *Material.* The material used in these experiments was obtained from the dark basement store rooms of a sugar refinery. No attempt was made to rear the cockroaches in the laboratory, but a fresh lot was secured each time it was desired to make a series of tests. They were always dark-adapted before they were used in the experiments.

2. *Methods.* A plain piece of slate 28 cm. square was used in exposing the cockroaches and thus the strong thigmotrophic response of these insects called forth by sharp corners or concave surfaces was avoided. The slate supported by a small table 16 cm. in height was placed inside a glass box 40 cm. square by 15 cm. in height. The purpose of the box, which in no way interfered with the rays of light, was to entrap the cockroaches when they ran off the edge of the slate. When a large number of specimens had thus accumulated they were re-collected but were not used in a second series of tests until they had become dark-adapted. The entire apparatus including the outside of the glass box was painted dead black to reduce to a minimum the reflection of any light.

A cockroach to be tested was placed at the middle of the surface of the slate, with its axis perpendicular to the direction of the rays. In this position there was an equal chance of its going to one side or the other unless influenced by the light. The direction of movement of the cockroach was not always directly towards or away from the source of light, but was frequently to one side of the illuminated field to the darkness. Some of the erratic movements of these highly excitable and nervous insects may be explained in part by what seems to be their intense fear while in the light. In such cases, where the orientation was not direct, the response was considered positive or negative according as the cockroach left the slate on the side toward or away from the source of light. If it left the slate on the side of the median line away from the light, the reaction was considered negative no matter if the course taken by the insect was in the direction of the rays or at an angle with them. If the response was in any direction toward the light it was counted as positive.

3. *Results.* Single monochromatic lights were used first to determine the relative effectiveness of each light in stimulating the cockroach. The direction of the lights was reversed with each ten trials, but for a matter of convenience the records are combined.

From the records of the experiments shown in table 11 it may be inferred that the cockroach is positive to blue, negative to green and yellow and indifferent to red, results which are borne out by experiments with balanced lights of different colors to be described later.

When paired lights of the same colors were used, there was practically an equal distribution of responses to the two sides.

The records in table 12 while of no value in determining the relative potency of the colors, do show the degree of reliability that can be placed on tests of cockroaches with balanced pairs of lights.

TABLE 11
Reactions of Periplaneta to single monochromatic lights

COLORS	NUMBER OF REACTIONS		PERCENTAGE OF NEGATIVE REACTIONS
	Negative	Positive	
blue.....	30	73	29
green.....	89	47	65
yellow.....	108	65	63
red.....	55	60	48

TABLE 12
Reactions of Periplaneta to pairs of monochromatic lights of the same quality and of equal intensity

COLORS		NUMBER OF REACTIONS		PERCENTAGE OF REACTIONS TO THE LEFT
Left	Right	Left	Right	
blue.....	blue	62	54	53
green.....	green	68	61	53
yellow.....	yellow	57	59	49
red.....	red	57	58	50

The results of the experiments with balanced monochromatic lights of different colors are represented in table 13.

In each record of table 13 where blue is compared with any one of the other colors or with darkness the majority of the cockroaches were oriented in a direction towards the blue. There is, however, no consistent gradation in the percentages of responses that corresponds to the spectral position of the color opposed to the blue.

In the contrasts of green or yellow with other colors the majority of the responses of the cockroach are negative to the green and to the yellow. The percentages of the responses in these cases are shown for greater convenience in comparison in table 14.

TABLE 13

Reactions of Periplaneta to pairs of monochromatic lights of equal intensity

PAIRS OF MONOCHROMATIC LIGHTS		NUMBERS OF COCKROACHES GOING TO EACH LIGHT		TOTAL NUMBER OF COCKROACHES TO EACH LIGHT		PERCENTAGE OF COCKROACHES TO MORE REFRACTIVE LIGHT
Left	Right	Left	Right	More refractive	Less refractive	
blue	green	86	41	165	85	66
green	blue	44	79			
blue	yellow	88	18	191	79	71
yellow	blue	61	103			
blue	red	71	33	166	74	69
red	blue	41	95			
green	yellow	52	56	109	112	49
yellow	green	56	57			
green	red	43	76	87	153	36
red	green	77	44			
yellow	red	45	86	89	170	34
red	yellow	84	44			

TABLE 14

Percentages of reactions of Periplaneta to each of the four colors and to darkness when compared with green and with yellow

COLORS	BLUE	GREEN	YELLOW	RED	DARKNESS
green.....	66	53	50	64	65
yellow.....	71	49	49	66	63

In the sets of pairs in which green and yellow are compared with the other colors the percentages of negative responses in each pair are practically equal. Thus the theoretical result when yellow is balanced with yellow or green with green is 50 % : 50 %, while the actual result is 53 % : 47 % and 49 % : 51 % for the green and yellow combinations respectively.

The percentages of responses when green-yellow and yellow-green are opposed are 49 % : 51 % and 50 % : 50 % respectively. These results indicate that yellow and green are practically equal in potency as far as their effectiveness in orienting the cockroach is concerned.

TABLE 15

Percentages of reactions of Periplaneta to each of the four colors when compared with red and with darkness

COLORS	BLUE	GREEN	YELLOW	RED
red.....	69	36	34	50
darkness.....	71	35	37	52

The reactions of the cockroach to the pairs of colors in which blue is balanced with green and yellow, the percentage of the responses to the blue is not greater than when blue is used alone. This result is somewhat inconsistent in view of the fact that the cockroach is negative to the green and yellow and positive to the blue. In using such pairs of lights one would naturally expect a larger percentage of the cockroaches to go to the blue than in the case when blue is used alone.

In table 15 are shown the percentages of responses for the pairs red or darkness with each of the colors. The results exhibited in this table demonstrate that red of an intensity and quality used in these experiments, has no more effect than darkness in stimulating the cockroach.

The reactions of the cockroach to light are unique in that they are positive to the blue, negative to the green and yellow and indifferent to red.

5. DISCUSSION

The results of these experiments with lights of measured intensity demonstrate conclusively that the effectiveness of the different colors of the spectrum does not correspond to the relative intensity of luminosity of the lights, as Merejkowsky ('81) has stated, but to their specific quality. Merejkowsky's method of equalizing the intensity of the lights, by judging the relative luminosity with his own eye, is very inaccurate, because the different colors of the spectrum have an effect on the eye which is not proportional to their energy content. Furthermore, the maximum brightness of the spectrum differs with different degrees of illumination as is well known from the Purkinje phenomenon. In the bright spectrum the region of greatest luminosity lies in the yellow, in a spectrum from a weaker source, it is in the green. Merejkowsky's results, therefore, cannot be considered seriously as opposing the view, that the efficiency of the different colors of the spectrum to stimulate the lower animals, is independent of intensity.

The view of Hess ('10) that the relative attractive power of the different homogenous lights approaches or corresponds with the brightness curve of the color-blind person, is not fundamentally different from the view held by Merejkowsky. Hess has shown, contrary to Loeb's hypothesis, that the reactions of animals are not the same as those of plants in their response to the spectrum, but he has not proven that the yellow and green have the greatest stimulating efficiency when the factor of intensity is eliminated. His experiments show that the yellow and green are more efficient than the orange and red of the spectrum, since the latter contain a much greater amount of radiant energy. As the yellow-green rays contain much more energy than the blue and violet, the seemingly greater effectiveness of the yellow-green rays is probably due to the greater energy of this region of the spectrum. The results of the experiments by Bert ('68) and by Lubbock ('81, '83) upon the reactions of *Daphnia* to the spectrum are similar to those obtained by Hess, but unfortunately these investigators also ignored the factor of intensity.

I do not mean to say that either *Daphnia* or many of the other arthropods or the other animals investigated by Hess are not more responsive to yellow-green rays than to those of shorter wave length, but I do maintain that in none of these experiments have the investigators proven that the yellow-green rays are more potent than the blue rays, when the factor of intensity is eliminated.

The results of the present investigations have shown conclusively that when lights of equal intensity are used, the adults of *Calliphora*, *Drosophila* and *Feltia* and the larvae of *Zeuzera* are more responsive to blue than to green or yellow.

The reactions of the animals named in the preceding paragraph agree with the statements of Loeb ('05) and of Davenport ('97) that the more refrangible rays of the spectrum are more effective stimuli than the less refrangible rays. But the hypotheses of Loeb and of Davenport are not in accord with the results of the experiments upon the *Calliphora* larvae, which are most responsive to the green rays of the spectrum. Loeb's hypothesis was based on the results of experiments which involved the use of only two colors, namely, blue and red. In his experiments upon larvae and other animals he did not compare the efficiency of the blue end of the spectrum with the green and yellow rays. Loeb therefore did not have sufficient evidence, as has been pointed out by other authors, on which to base such a general conclusion.

In order to have an unobjectionable basis for comparing the results of the experiments upon *Calliphora* larvae with those on adult insects under the blue-green combination of lights, the adult *Calliphora* and also adult *Drosophila* were each tested with the same lights and adjustments that were used in taking a series of records similar to those shown in figures 15 and 18 and immediately after the observations on the larvae. Out of 37 adult *Calliphora* used in five tests, 24 went to the blue and only 13 to the green; likewise in a second check experiment out of 42 *Drosophila* 33 went to the blue and only 9 to the green. These tests demonstrate that the adult flies are more responsive to the blue under conditions of illumination identical with those in

which the *Calliphora larvae* are more affected by the green rays and answer any possible objection that the results shown in the records were caused by slight differences in the apparatus, intensities, et cetera, employed for the larvae and for the adults. This experiment proves that the relative stimulating efficiency of the rays of different colors is not the same for all animals nor for different ages of the same animal, and that the more refractive rays are not always the most effective.

Mast ('11) in developing the same idea, cites as evidence the following investigations among others: Wilson ('91), who found hydra to be more responsive to blue than to violet; his own work ('00) on amoeba, an animal which is also affected most by the blue; the work of Bert ('68), Lubbock ('81 a, '83) and Yerkes ('99, '00) on *Daphnia*, a crustacean, which aggregated in the yellow-green region of the spectrum; and the investigations of Engelmann ('82), who discovered that *Bacterium photometricum* collected in the infra red of the spectrum. These investigations do not prove that hydra and amoeba are affected most by the blue rays, the *Daphnia* most by the yellow-green and *Bacterium photometricum* most by the infra red independent of the intensity factor. They do show, however, that the animals tested respond differently in degree to the various rays of the spectrum, since presumably the spectrum used was similar in each case. If the results of these investigations are reliable, then, in the same spectrum amoeba and hydra are affected most by the blue, *Daphnia* most by the green-yellow and *Bacterium photometricum* most by the infra red. This work, therefore, in spite of the fact that intensity was ignored, supports the view that the stimulating efficiency of the rays of different wave lengths is not the same for all animals.

The reactions of *Calliphora larvae* to colored lights are similar to those exhibited by these larvae to white light, as demonstrated by Herms ('11). The larvae, when opposing balanced colors of the same wave length are used, crawl, after orientation, in a direction approximately perpendicular to the direction of the rays, as I have shown in the observations presented in this paper. If lights of the same intensity but of different wave lengths

are used the larvae proceed at an angle to the direction of the rays, as they do when they are stimulated by opposed white lights of unequal intensity; but if the efficiency of one of the lights is much greater than that of the opposing light, as, for example, blue against red, the larva is oriented directly towards the latter by the dominating stimulus of the more potent color.

The larvae of *Zeuzera pyrina*, which are positively phototropic, never crawl at an angle to the direction of the rays, but, after once having been oriented, proceed directly toward the source of light. When paired lights of the same wave length are used the larvae instead of crawling at right angles to the direction of the rays, as the *Calliphora* larvae do, go directly to one or the other of the two sources of light. When the lights are of different wave lengths, the larvae are oriented by the more potent color and proceed in a direction approximating that of the rays. The reactions of *Zeuzera* agree with the general statement made by Loeb ('05, p. 82) concerning the reactions of animals to white light from two sources: "If there are two sources of light of different intensities, the animal is oriented by the stronger of the two lights." Loeb's statement applies to the reactions of *Zeuzera* larvae but it certainly does not hold for the reactions of *Calliphora* larvae nor for that of many other animals, a criticism of Loeb's work which has also been expressed by others. Adults of *Drosophila* and *Calliphora*, insects with image-forming eyes, creep or fly toward the lights and, like *Zeuzera* larvae, when balanced lights of different wave lengths are used, are oriented by the more potent color. In all of these tests with adult flies, however, a certain percentage of the individuals go away from the source of the light which to the majority of the flies is the most effective stimulus. It is difficult to explain this difference in organisms of the same species, reared under identical conditions and tested with the same lights. To say it is due to a difference in physiological condition brings us no nearer to the solution. It is evident from the results shown in table 8 that the flies which go away from the more effective light at the first trial do so not because of a permanent difference in their phototropism, but because of mere chance or some unknown

factor operating in their initial orientation. If the fly on entering the chamber illuminated by opposing colors of nearly the same wave length (as, for example red and yellow) is headed directly away from the more effective light, the chances are it will remain under the influence of the less potent light, since more of the elements of the compound eyes are effected by this light than by the other. In a case where there is a great difference in the efficiency of the two opposing lights (as, for example, when red and blue, or blue alone is used) the more refrangible rays, though impinging upon relatively few elements of the eye, are so effective that the insect will be oriented towards the blue light even when it enters the cylinder facing away from the more potent light, or in a direction oblique to the direction of the rays.

The reactions of *Periplaneta americana* in response to colored lights of equal intensity are remarkable in being positive to blue and negative to green and yellow. It is not an uncommon occurrence to produce a reversal of phototropism among the lower organisms by changing the nature of their environment or by using extreme differences in the intensity of illumination, but I know of no recorded observation in which an animal has been shown to be positive to one monochromatic light and negative to another of the same intensity, but of different wave length.

Graber ('83), working with a closely allied species of cockroach, *Blatta germanica*, found that these insects collected on the red side of a two-chambered compartment illuminated respectively with blue and red sheets of glass. From these results one would infer that *Blatta* is negative to blue, not as Graber ('84, p. 152) interprets a "blauscheues resp. rot-holdes Insect." No one has ever verified with spectral light Graber's results on the cockroach so the discrepancy in our results is in all probability due to difference in method. The blue glass used by Graber transmitted not only the blue rays but red and green, some yellow, and the invisible heat rays. Furthermore Graber did not state in just what manner the aggregation of *Blatta* took place.

At the present state of our knowledge, it is difficult to offer an explanation of the reversal of the phototropism of the cockroach to the different colors.

If it is assumed that the reactions of *Periplaneta* are brought about by chemical changes produced by light on the photoreceptors of the organism, it is conceivable that there exist within the organs photo-chemical substances, not unlike triphenylfulgid, which have reversible reactions. In blue or violet triphenylfulgid changes from orange-yellow to a dark brown compound; in the red and yellow rays this reaction is reversed. Though no such substances are known to exist in the body of the cockroach, it is nevertheless an interesting suggestion and when our knowledge of the chemistry of the reactions of animals to light is better known, it is possible that explanations of such complicated reactions as those of *Periplaneta*, as well as those of other organisms, may be easily explained.

6. SUMMARY

1. The larvae of *Calliphora* and *Zeuzera* and the adults of *Calliphora*, *Drosophila*, and *Feltia* are responsive to each of the four colors of the spectrum used in these investigations.

2. The colors in the order of their efficiency in stimulating the larvae of *Calliphora* are beginning with the most effective: green, blue, yellow, red.

3. The order of the effectiveness of the colors in stimulating the larvae of *Zeuzera* and the adults of *Calliphora*, *Drosophila*, and *Feltia* is the order of the natural sequence of the colors in the spectrum: blue, green, yellow, red.

4. The relative stimulating efficiency of the rays of any part of the spectrum is independent of intensity, and is not the same for all animals nor for different ages of the same animals.

5. The more refractive rays of the spectrum are not always the most effective in stimulating an organism.

6. *Periplaneta americana* is responsive to blue, green and yellow, but is indifferent to red. It is positive to blue and negative to both green and yellow. Green and yellow are practically equal in their stimulating efficiency, as far as the reactions of *Periplaneta* are concerned.

7. There is a reversal of phototropism in the reactions of *Periplaneta* to the different colors of the spectrum which, as far as the tests in these investigations indicate, is independent of the intensity of the light and of any chemical condition of the environment.

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tions at first showed radiating strands of sperm visible to the naked eye, but then closed to form a solid drop, and this grew at its margins, preserving the clear external zone, until the clear margins of the two drops, at first several millimeters apart, ran together. Outside of the influence of the drops cloud formations appeared, corresponding to an early stage of the aggregations of *Nereis*.

The clear margin of the drops is not by any means so well defined as in *Nereis*. Moreover, the spermatozoa are so small that it is difficult to observe their behavior in the clear margin. However, there can be no doubt that the phenomenon is essentially the same as in *Nereis*, and that the aggregation in the drop, the appearance of the clear margin, and the growth of the aggregation are due to positive chemotaxis to CO_2 .

This reaction is given clearly only by a fresh sperm suspension. One ten minutes old does not give it, owing presumably to formation of CO_2 in the suspension.

A considerable number of tests were made. In some the reaction was much more rapid than in the experiment described. In one of these tests I injected drops of 20 per cent, 4 per cent and 1 per cent of the CO_2 sea-water near together. In the case of the 20 per cent a ring with external clear margin was formed in a few seconds. The ring did not close. The 4 per cent formed a ring which closed in its center. There was no reaction to the 1 per cent. The same suspension gave no reaction twenty-five minutes after it was mixed. In another case I got a faint reaction to 1 per cent CO_2 sea-water.

The fact of positive chemotaxis of *Arbacia* sperm to CO_2 dissolved in the sea-water was repeatedly demonstrated. In the case of a drop mounted beneath a raised cover it expresses itself by a gradual aggregation of the sperm towards the center, leaving the margins clear.

As is to be expected from the slower and less delicate reaction to CO_2 , as compared with *Nereis*, spermatozoa of *Arbacia* react also to other acids, but more slowly and not to so great dilutions. Thus in tests of N/10, N/50, N/250 and N/1000 H_2SO_4 , strong positive reactions were obtained for the first three, whereas only a faint shadowy reaction is given to N/1000. In the case of *Nereis*

it will be recalled that N/2000 gives a distinct reaction. Positive reactions were not secured with N/1000 HCl or HNO₃, but were with N/250. The ring formed in response to N/250 H₂SO₄ grows somewhat owing to diffusion of the acid; in the case of N/50 and N/10 H₂SO₄ the growth is much greater, in general in proportion to concentrations.

As in Nereis alkalis agglutinate, but cause no aggregation. KOH was tested in two ways: (1) addition of a small quantity of N/2000 KOH in sea-water to a vial of fresh sperm suspension caused macroscopic agglutinations which are irreversible. (2) a drop of N/2000 KOH injected into a suspension beneath a raised cover fills in a short time with sperms that agglutinate; but there is no chemotaxis, and in a short while the drop is left to one side by aggregation of the sperm away from it, owing to rise of CO₂ tension elsewhere.

Reaction to egg-secretions

As contrasted with the slowness of reaction of Arbacia spermatozoa to acids, the reaction to egg-secretions is instantaneous and clear cut. There is a most pronounced positive chemotaxis, as tested with drops even of very weak egg-extract injected into a sperm suspension mounted beneath a raised cover-slip; but this is always associated with agglutination, and is, therefore, best considered under that head.

IV. AGGLUTINATION PHENOMENA AND REACTIONS OF SPERMATOOZOA TO EGG-SECRETIONS

1. INTRODUCTION

Following the demonstration of definite chemotactic behavior of spermatozoa of Nereis and Arbacia the question naturally arises what relation, if any, has this form of behavior to the union of egg and spermatozoon in fertilization. As is well known, the mere observation of the interaction of the sexual elements in fertilization led long ago to the theory that the egg attracts the spermatozoon to itself by chemotaxis, and fusion results from active penetration of the ovum by the spermatozoon. But in recent years there has been a tendency to deny both of these

principles as factors in the union. Chemotaxis has fallen into disrepute; and the theory that the spermatozoon bores into the egg has been rejected by several observers.

If chemotaxis is concerned in the union of ovum and spermatozoon the medium in which fertilization operates must contain the substance concerned. In the case of the eggs of *Nereis* and *Arbacia*, therefore, the hypothetical substance which attracts the spermatozoa must exist in sea-water which has been in contact with fertilizable eggs; and it must be possible to obtain a sufficient concentration of the substance in question in sea-water to demonstrate its presence by reaction of the spermatozoa, because, *ex hypo.*, the substance exists in effective amounts in the sea-water surrounding the eggs. If it were impossible to demonstrate the presence of an agent to which spermatozoa of the same species are positively chemotactic by such means the theory of chemotaxis would have to be abandoned. However, the presence of such a substance is readily demonstrated both in *Nereis* and *Arbacia*.

In the second place, if the union of the ovum and spermatozoon after they have come in contact operates not mechanically, but through some bio-chemical reaction between spermatozoon and ovum, the sea-water in which eggs have been standing should contain a substance also capable of reaction with the sperm, which should be an efficient indicator for it.

I was guided by some such ideas as these in the series of experiments which follow, and which showed at the very first trials that sea-water which has stood with fertilizable eggs of *Nereis* or *Arbacia* contains a substance to which the spermatozoa of the same species are positively chemotactic, and also a substance which agglutinates the spermatozoa of its own species. It may be that one substance is concerned in both reactions, but it is more probable that two are present. It is perhaps worth emphasizing here, for this is the fact that struck me at the start, that the sea-water which has stood over eggs³ combines both the effects of

³ To avoid the frequent repetition of such a circumlocution we may call sea-water, which has contained eggs and is charged with their emanation, egg sea-water, and the concentration of the substance in the sea-water may be expressed by writing the relative bulks of eggs and sea-water as a fraction. Thus 'egg sea-water 1/3' would indicate that the bulk of eggs was one-third the volume of the sea-water. Time is also a factor in the concentration, of course.

an acid (aggregation) and also an alkali (agglutination) on the spermatozoa. The comparison may, of course, be superficial, but it serves at least to emphasize the double action of the egg-secretion.

As contrasted with the difference in rapidity and delicacy of reaction between the spermatozoa of *Nereis* and those of *Arbacia* to inorganic substances, we may note in advance that the reactions to the egg extractives are as rapid and clear in the one case as in the other, and are entirely similar in principle, though there are certain secondary differences that will be noted in the proper place.

2. INITIAL EXPERIMENT

We may begin by describing the reactions to be observed in the case of an *Arbacia* sperm-suspension freshly made and mounted beneath a raised cover-slip, into which a drop of *Arbacia* egg sea-water 1/10 to 1/20 about half-an-hour-old is injected. The naked eye observation shows almost instantaneous formation of a ring at the margin of the drop, with simultaneous formation of a clear external zone about 1.5 to 2 mm. wide; the ring then breaks up into small agglutinated masses and so becomes beaded. The trail of substance left in withdrawing the pipette extends to the margin of the cover-slip. It also is a center of attraction and the ring is therefore prolonged by a chain of agglutinated masses to the margin.

One can observe the details of the reaction best under the microscope, using a low power, by bringing the point of the pipette into the field of the microscope and blowing in the drop with the aid of a flexible rubber tube held in the mouth, while looking through the microscope. The reaction takes place so rapidly that it requires repeated observations to observe all the details. In the first second the spermatozoa are aroused to intense activity and form small agglutinated masses within the drop; these then appear actually to 'rush' together (to use the language of my note book) to form larger agglutinations for a period of three to five seconds, after which no more fusion of masses takes place. The agglutinated masses thus range from relatively large to relatively small. While this has been going on in the interior of the drop, a ring

has formed at the margin, and a clear zone arises external to it. The ring is at first continuous, but it ruptures in numerous places in two or three seconds and each segment contracts quickly to an agglutination mass.

The agglutinated masses in the interior of the drop are smaller than in the ring, owing to the relatively low concentration of the sperm suspension within the drop, and they break up very quickly while the sperm is still extremely active. The movements of the spermatozoa then gradually slacken; in a few minutes the larger and more firmly agglutinated masses of the ring also begin to break up and in ten or fifteen minutes all are resolved.

This preliminary observation demonstrates a three-fold action of the egg-extractive: (1) it activates the spermatozoa; (2) it aggregates them through positive chemotaxis; (3) it agglutinates them. The phase of increase of activity lasts only a short time, a minute or two at the most, after which movements of the sperm slacken and become less than the control, or cease entirely. Positive chemotaxis (aggregation) is shown by formation of a clear zone external to the marginal ring. This is always a sign of chemotaxis, as we have seen in the preceding section.

The agglutination phenomenon is fundamentally different from the aggregation; in the latter the spermatozoa are merely loosely associated, and slight agitation is sufficient to scatter them. In the agglutinated masses the spermatozoa are stuck together and are not separated by shaking. In the case of *Nereis* where the agglutination is firmer than in *Arbacia* the masses may be broken up into smaller masses by needles, or preserved *en masse* in killing fluids. The breaking up of the ring into separate masses is a characteristic agglutination effect; the rings formed in response to an acid do not break up unless the acid is very weak (see p. 537). Finally an agglutinative substance produces its effect when shaken up and evenly distributed in a vial of sperm suspension, but an aggregative substance cannot of course exert a chemotactic effect in the absence of a gradient.

The same experiment succeeds well with *Nereis*. The eggs of this form give off a substance (or substances) into the sea-water, which causes aggregation and agglutination of the spermatozoa

when a drop of sea-water so charged is injected into a fresh sperm suspension beneath a raised cover slip. The activation is not so pronounced in this case as in *Arbacia*. The aggregation phenomenon is the same. The agglutinations are substantially permanent in *Nereis*; the spermatozoa stick together much more firmly.

In what follows we may leave the activation and aggregation phenomena out of account for the most part and confine ourselves to the problems of agglutination. The substance which causes agglutination of the spermatozoa we shall call the sperm agglutinin. The agglutination may be shown very strikingly in a vial of fresh sperm suspension. In the case of *Arbacia* the addition of two or three drops of egg sea-water 1/4, which has stood half-an-hour, to about 2 cc. of a fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half-an-hour or more.

The degree of agglutination is of course dependent on the density of the suspension. This is shown by the following experiment: Ovaries and eggs of *Arbacia* were cut up in about four times their own bulk of sea-water and allowed to settle. Two cubic centimeters of the supernatant fluid was put in each of three vials. To one was added 3 drops of a fresh milky sperm suspension, to the next 12 drops of the same, to the third 36 drops. No visible agglutinations formed in the first; in the second agglutinations became visible to the naked eye almost immediately, in the third agglutinations were larger, more numerous, and apparently more solid.

3. OVA ALONE PRODUCE THE AGGLUTINATING SUBSTANCE

The eggs of both forms thus produce an agglutinin in the sea-water. The next question is whether the agglutinin is specifically an egg-product. A considerable number of experiments prove that this is the case. The large body cavity of *Arbacia* is filled with abundant coelomic fluid and this may be supposed to

contain substances from various tissues. But it invariably proved perfectly neutral to spermatozoa of *Arbacia*, even when taken from females with large ovaries, showing that the substance concerned in agglutination does not escape from the ovaries of the intact animal, or, if it does, that it is promptly destroyed. Nor was it possible to extract a sperm agglutinating substance for *Arbacia* by extracting the intestine in sea- or fresh-water.⁴ These experiments were repeated a sufficient number of times to be conclusive.

As illustrations: (1) Intestine extractives. August 31, 1912. The intestines of several *Arbacia* were cut up in about twice their bulk of distilled water, and were allowed to stand in it about an hour. The strongly amber-colored fluid was filtered off and rendered isotonic with the sea-water by addition of concentrated sea-water (four parts of the latter to six of the intestine extract). This fluid causes no agglutination in sperm suspensions. A similar extract of the ovaries caused immediate large dense agglutination masses. (2) Coelomic fluid: As is well known, the coelomic fluid of *Arbacia* contains large numbers of densely pigmented corpuscles. Outside the body the fluid quickly forms a loose clot which includes many of the corpuscles. The others can be separated from the remaining serum by centrifuging. The corpuscle-free serum was sometimes used, sometimes simply the clot-free serum. Repeated tests were made both by injecting drops into fresh sperm suspensions beneath raised cover glasses, and also by mixing with fresh sperm suspension in vials. Whether the coelomic fluid came from males or females it proved invariably negative, except for the faintest sort of agglutination reaction in one or two cases only, which may have indicated some individual differences. It is interesting in view of these facts that outside of the body the coelomic fluid becomes heavily charged with the sperm agglutinin if eggs are placed in it. It must be supposed therefore that the ovarian membrane is impermeable to the agglutinating substance in the intact animal.

⁴ The experiments this year simply opened up the problems, and it was impossible to make any quantitative tests or adequate chemical examination. For the purpose of the biological problem of the behavior of the spermatozoa with reference to the eggs the question of immediate importance was the behavior with reference to egg-extractives or secretions in the sea-water tested.^{*} Stronger agglutinating solutions were made by increasing the quantity of eggs with reference to the sea-water, or by crushing the eggs in sea-water. Distilled water was shown to extract more agglutinin from a given bulk of eggs than sea-water; and the coelomic fluid of *Arbacia* also proved to be a better medium for extracting agglutinin from the eggs than sea-water. It would be possible of course to establish quantitative values for all of these relations, and this problem should receive attention. The problems of solubility of the agglutinin in various media, and other chemical questions, also present themselves.

In the case of *Nereis* it was not possible to reach such conclusive results, because the sexually mature female is practically a bag of eggs, and one cannot obtain other organs for testing. I cut up five females that had shed their eggs in 10 cc. of sea-water. In spite of efforts to get rid of all eggs, a considerable number were in the water. For control I used the eggs of two females in 100 cc. sea-water. On test in half-an-hour the fluid from above the eggs was found to be about ten times as agglutinative as the fluid from the bodies of the spent females. So that it is certain that other tissues do not produce much sperm agglutinin and it is probable that they do not produce any. The small amount present could be accounted for by the few eggs included, and perhaps by egg secretions absorbed by the tissues.

4. FIXATION OF THE AGGLUTININ BY SPERMATOZOA

The next question was whether the agglutination reaction as described has the usual characters of a chemical reaction? The general result is (1) that an agglutinated sperm suspension in which reversal has occurred is not capable of re-agglutination by addition of more of the agglutinating substance and (2) that the agglutinating substance disappears from an agglutinated suspension if not present originally in excess.

As regards the first point, the earlier experiments were concerned entirely with the form and conditions of the reaction, and the agglutinating substance was always used, as later results showed, in excess. It was not possible to get a repetition of the agglutination reaction under these circumstances. But one can get a repetition of the reaction in a sperm suspension by addition of successive small amounts of the agglutinating substance, until the reaction is complete, as the following experiment shows:

September 4, 1912. *Arbacia*. 2 cc. of a creamy active sperm suspension was agglutinated with 5 drops of an egg-extract prepared as follows: The ovaries of three females were cut up in about three times their volume of distilled water and allowed to stand about thirty minutes. Then the water was filtered off and made isotonic with sea-water by the addition of concentrated sea-water (proportions of 58 to 42 parts). This made a very strong agglutinating extract. After reversal of the agglutination described above the agglutination was repeated by addition of a drop of

another egg-extract. This time the agglutination was complete for it could not be repeated a third time. Other sperm suspensions gave similar results.

The result might be interpreted as a purely biological reaction, that is to say in terms of stimulation, were it not for the fact that the agglutinating substance disappears from an agglutinated sperm suspension, as shown by the following experiment:

September 12, 1912. *Arbacia*. Nine parts of a thick active sperm suspension was agglutinated by one part egg-extract. The agglutination produced was so strong that the fluid between the white masses appeared clear to the eye. In three or four minutes reversal of the agglutinations had begun. The agglutinated sperm suspension was then centrifuged until practically all the sperm was precipitated. The supernatant fluid was tested and agglutinin was shown to be absent. As control, a dilution of one part of the same egg-extract with 9 parts of sea-water was tested with the same sperm and proved to be strongly agglutinative. Three tests were made with each with uniform results.

There can be no doubt, as the result of this and other observations also, that the spermatozoa fix in some way the agglutinating substance, and it will be simplest to assume as a working hypothesis that the fixation is due to chemical union. I have not yet had the opportunity to ascertain if the agglutinin could be regained from the sperm precipitated in the centrifuge.

5. NATURE OF THE EFFECT ON THE SPERM

We have noted four effects of the egg-extracts on sperm of the same species, namely: (1) Stimulation of intense activity, which is of brief duration. This is more marked in the case of the sperm of *Arbacia*, than in the case of the naturally extremely active sperm of *Nereis*; (2) An orienting effect expressed in positive chemotaxis; (3) An agglutinating action; (4) Following these effects more or less complete paralysis of the sperm.

To what kind of change in the individual spermatozoa is the agglutination reaction due? We may note in the first place that the agglutination is between the heads of the spermatozoa, and that the tails are apparently unaffected, at least at first; it is only in later stages of the action of the agglutinating substance that the locomotor function is injured. The adhesion of the heads

demonstrates some change in the membrane that renders them sticky. The cells are so minute that it is difficult to observe any microscopical change in the case of *Arbacia*; in *Nereis* the spermatozoa are larger, and it can be seen that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one, indicating a great increase in permeability. The spermatozoa which have undergone this change are usually motionless, and, when not fused with one another, appear to be glued to the slide or cover slip, never freely suspended.

Agglutination in itself is in no sense a specific reaction, but one that may be expected to accompany certain superficial changes of the spermatozoa, however caused, under conditions that bring the spermatozoa into contact. It occurs, to a limited extent, spontaneously in sperm suspensions that have stood for some time. It is particularly noticeable in *Nereis* under the following conditions: A fresh sperm suspension is allowed to aggregate on a slide beneath a raised cover slip and the aggregations remain undisturbed. In the course of ten or fifteen minutes small agglutinated masses may form around the margins of the aggregations or beneath the aggregations in contact with the slide. There may be twenty to fifty or more such masses associated with a single aggregation, and they are quite similar in their general appearance, to those produced suddenly by the agglutinin of the egg, though much smaller, on the average.

It is not probable that the agglutination is in any real sense toxic or cytolytic. It is true that the agglutinin inhibits movement after a few minutes, and it certainly lessens the fertilizing power of the sperm. But if an agglutinated mass of spermatozoa of *Nereis* be crushed under the microscope many of those liberated are active. Moreover, if a small quantity of an agglutinated suspension of spermatozoa be added to a relatively large quantity of sea-water fertilizing power is partially regained. This might be either because some of the spermatozoa of the agglutinated suspension had escaped combination with the agglutinin and were alone concerned in the actual fertilization, or because of recovery

from the agglutination effect. It is very difficult to form a definite opinion as to the real nature of the agglutination effect.

6. THERMO-RESISTANCE OF THE AGGLUTININ

The agglutinating agent is slowly destroyed at 95°C.:

August 28. *Arbacia*: (1) Ovaries and eggs of *Arbacia* were cut up in three times their bulk of sea-water, and let stand about an hour. The supernatant fluid is strongly agglutinative on *Arbacia* sperm suspensions. (a) Part of it was now taken and boiled about thirty seconds, and cooled. On test it proved as agglutinative as before. (b) Some more was then boiled five minutes and cooled. Its agglutinative power was apparently undiminished. (c) Another egg-extract similar to the first was then boiled and put in a beaker of boiling water for thirty minutes; the temperature stood about 95° during this process. On test its agglutinative power was shown to be greatly diminished. (d) In a fourth test some egg-extract was kept at 95° for sixty-six minutes. It still exhibited some agglutinative power, which, however, was very slight as compared with the control. The heated egg-extract exhibited a considerable change of color from the yellowish red of the control to a much brighter red.

Simultaneously with the loss in agglutinating power, it appeared also to gain in aggregating power: Drops of the heated and the unheated egg-extract were injected into the same sperm-extract beneath a raised cover. The drop of the heated expanded its sphere of influence shown by immigration of the sperm, about twice as rapidly as the unheated; this was tested several times; so that it would appear that the aggregative and agglutinative agents are probably distinct, and that the agglutinin inhibits aggregation to a considerable extent.

Nereis: The eggs of three females were inseminated in 9 cc. sea-water; the supernatant fluid has a slight amberish-green color, and is strongly agglutinative on *Nereis* sperm. (a) After keeping at 95° C. for ten minutes its agglutinative power was much reduced. (b) After twenty-two minutes at 95°C. the agglutinin was entirely destroyed. The color was entirely destroyed also.

Thus the agglutinating substances, whatever they may be, are either volatile being gradually driven off by heating, or they are slowly coagulated or disintegrated chemically by a temperature of 95°C. The agglutinin of *Nereis* is either more volatile or more labile than that of *Arbacia*. It is impossible to say definitely to what class of chemical substances these agglutinating

substances belong. It is, however, extremely improbable that they possess a degree of chemical simplicity sufficient to allow of volatilizing; it is more probable that they undergo slow chemical disintegration at the temperature employed. The thermostability of these sperm isoagglutinins is relatively very high, and this perhaps makes it doubtful whether they can belong to the same class of substances as the haem-agglutinins of vertebrate blood sera. This matter must therefore remain undecided, and it should be understood that the term agglutinin is used in the present paper in a purely descriptive sense.⁵

7. FERTILIZING POWER OF AGGLUTINATED SPERM

The powerful effect of the egg-extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power. The following experiments illustrate this:

1. *Arbacia*. The egg-extract used was made by cutting up the ripe ovaries in about three times their bulk of distilled water; in half-an-hour the water was filtered off and was then made isotonic with sea-water by the addition of 42 parts of condensed sea-water to 58 of the egg-extract. Five small watch crystals in a series contained (1) 8 drops of the egg-extract (2) 4 drops egg-extract, + 4 drops sea-water (3) 2 drops egg-extract, + 6 drops sea-water, (4) 1 drop egg-extract, + 7 drops sea-water (5) 8 drops sea-water. To each of these 3 drops of opalescent sperm suspension was added; and after twelve minutes a drop of a suspension of fresh eggs was added to each. We thus had the same quantity of eggs in sperm suspensions of the same density, but in graded amounts of egg-extract. The sperm suspensions were so dense that in the control (no. 5) the jelly became packed with sperm, forming dense halos around the eggs. In (4) a very few (about 1 per cent) had slight halos of sperm in the outer layer of the jelly, but in (1), (2) and (3) the paralysis of the sperm was so complete that they did not enter the jelly of the eggs at all. Ninety-seven minutes later none of the eggs in (1), (2), (3) or (4) had segmented; whereas at least 5 per cent of the control were now in the two-celled stage. The lot of eggs was rather poor in this case, but fertilization was confined entirely to the control.

If the experiment be made in another way some recovery of the spermatozoa from their state of paralysis may be observed. Thus: An

⁵ It should be borne in mind that but little is known concerning lysins or agglutinins of invertebrates. It is perhaps not to be expected that they should exhibit the same degree of thermostability as those of vertebrates.

active sperm suspension was divided in two parts, and one part was agglutinated by the addition of about 40 per cent of its own volume of the egg-extract described above, to the other an equal amount of sea-water was added. The first was strongly agglutinated; after reversal both suspensions were stirred up, and beginning thirteen minutes after agglutination a series of fertilizations were carried out by adding one drop of the agglutinated sperm suspension to a measured quantity of eggs in about 9 cc. of sea-water at 10 minute intervals. Each fertilization had a control of the same quantity of eggs fertilized with one drop of the control sperm. The consistent result was that about 16 per cent of the eggs fertilized with the agglutinated sperm segmented and at least 33 per cent of the control. The non-agglutinated spermatozoa are about twice as effective as the agglutinated. But a considerable degree of recovery of some spermatozoa of the agglutinated suspension is shown.

2. *Nereis*. Experiments with *Nereis* did not give such a marked reduction of the fertilizing power of agglutinated sperm as in *Arbacia*. There was, however, a marked delay in the formation of jelly when agglutinated sperm was used as compared with normal sperm.

It is somewhat difficult to make a satisfactory interpretation of the effect of agglutination on fertilizing power. On the one hand we may suppose that a certain proportion of spermatozoa resist the agglutination effect, and are alone concerned in any fertilizing power of an agglutinated suspension; on the other hand it might be supposed that the agglutinating effect does not modify fertilizing power except as it decreases the motility of the spermatozoa or that the effect is reversible under the condition of the experiments. Either assumption would be consistent with the facts.

8. CONDITIONS OF FORMATION OF THE AGGLUTININ BY THE EGGS

The conditions of excretion of the agglutinating substance by the eggs into the sea-water is quite different in the two forms. In *Arbacia* the agglutinin is excreted continuously by unfertilized eggs in such amounts that I have not succeeded even by repeated washings in removing it all. Fertilization does not appear to increase or decrease the quantity. In *Nereis*, on the other hand, unfertilized eggs secrete but little of it, and one or two washings in sea-water will completely remove it so that the eggs secrete no more in detectable quantities; but at the moment of fertilization, on the other hand, it is poured forth in advance of the jelly in

large quantities and the eggs then appear to have disposed of their entire store, for washed fertilized eggs no longer produce it. The conditions are important in their bearing upon the question of permeability of the egg-membrane with reference to fertilization. The spermatozoa are very efficient indicators of substances leaving the egg. In the case of *Nereis* it can be shown that there is a sudden increase of permeability at the moment of fertilization, but in *Arbacia* such evidence is lacking.

The conditions in *Arbacia* may be shown by the following experiment: Eggs were washed free from all fragments of ovary and placed in about 20 times their own bulk of sea-water and divided in three lots, *a*, *b* and *c*. The sea-water over them agglutinates *Arbacia* sperm instantaneously. This test was made at 9.30 A.M. The supernatant fluid was then removed and the eggs washed in 20 times their bulk of sea-water. At 9.53 the supernatant water was tested and found agglutinative. 9.58, *a* and *b* were again washed. 10.04, supernatant fluid again agglutinative. 10.05, lot *a* was fertilized with 2 drops of sperm. 10.10, *a* and *b* tested again; both agglutinate. 10.16, *a* and *b* washed again. 10.23, supernatant fluid of both agglutinates. 10.30, *a* and *b* washed again. 10.36, both agglutinate sperm; *b* is rather more effective. 10.40, washed *a* and *b* again. 10.45, both agglutinate; *b* more effective. 10.51, washed *a* and *b* again. 11.00, both agglutinative; *a* more than *b*. 11.08 to 11.20, other tests of *a* and *b* show *a* somewhat more effective.

The experiment shows both fertilized and unfertilized eggs of *Arbacia* to be constantly secreting a substance into the sea-water which agglutinates the spermatozoa. The substance must be effective in very minute quantities for the amount of water used in each of the eight washings was at least ten times the bulk of the eggs, yet as soon as the eggs had settled to the bottom of the vials used the supernatant fluid contained the agglutinin in appreciable amounts. How long this process keeps up in *Arbacia* I cannot say; and too much reliance cannot be placed on the result for the fertilized eggs because a large proportion of the eggs failed to fertilize or at least to segment.

The conditions in *Nereis* are quite different; the experiments showed that shortly after the eggs are taken they charge at least ten times their bulk of sea-water with an easily detectable amount of sperm agglutinin. But if the eggs are washed once usually no more agglutinin can be demonstrated. If now the eggs are stirred

up in the vial, fertilized and allowed to settle, the supernatant fluid is very powerfully agglutinative. The experiment was repeated a sufficient number of times to make certain of this result.

A number of tests were also made with the object of determining if the eggs of *Nereis* continued to produce agglutinin after fertilization. These showed that the eggs cease very quickly their production of agglutinating substance. None could be detected during the maturation period, but apparently there is a second production about the time of the first cleavage. On this point I wish to be understood to speak with reserve. The swelling of the jelly secreted at fertilization makes the eggs very bulky, and the jelly itself takes up any egg secretion, so that there are considerable technical difficulties in making satisfactory tests.

The fact that stands out perfectly plainly in the case of *Nereis* is the sudden increase in secretion of agglutinin into the seawater just after insemination, followed by cessation of its production. The spermatozoa give absolutely positive tests. As will be shown later, the observations on the normal fertilization are in complete harmony with this.

The difference between *Nereis* and *Arbacia* in these respects is thus sharply marked. However, it should be remembered that the unfertilized eggs of *Arbacia* have formed both polar bodies, whereas those of *Nereis* are in the stage of the germinal vesicle. It may be that eggs of *Arbacia* in the germinal vesicle stage are relatively impermeable in the same sense as those of *Nereis*.

9. HETERO-AGGLUTINATION AND THE QUESTION OF SPECIFICITY: REACTIONS BETWEEN NEREIS AND ARBACIA

The demonstration of intraspecific sperm-agglutinating substances derived only from the ova having been made, the question arose whether these substances were essentially the same in both species, or different. If the same, the egg-extract of each should agglutinate the sperm of the other. A number of tests were therefore made which demonstrated conclusively that the substances are decidedly different with reference to their cross-agglu-

tinating effects, and this result has raised a number of questions, the most important of which relate to the question of specificity, but which could only be defined in the time at my disposal and not definitely answered.

The first suggestion that the sperm agglutinating substances of *Arbacia* and *Nereis* are different came from the following experiment: A raised coverslip preparation of *Nereis* sperm was made in the usual way and into it were injected a drop each of (1) *Arbacia* egg-extract in sea-water, (2) drop a of coelomic fluid from a female *Arbacia*, (3) a drop of coelomic fluid from a male *Arbacia*. All three caused very extensive firm agglutinations of the *Nereis* sperm.

Thus the egg-extract of *Arbacia* contains an agglutinating substance for the *Nereis* spermatozoa as well as for its own; but the coelomic fluid of *Arbacia* also causes agglutination of the *Nereis* spermatozoa, whereas it is perfectly neutral with respect to its own. This demonstrated, therefore, the existence of at least two sperm-agglutinating substances in *Arbacia*, namely: One in the egg-extract agglutinative for its own sperm and that of *Nereis*, and one in the coelomic fluid not agglutinative for its own sperm, but agglutinative for the foreign sperm of *Nereis*. The probability is, therefore, that the egg-extract contains both substances seeing that it is agglutinative for both kinds of spermatozoa. This was afterwards demonstrated.

The reciprocal experiment proved the difference of the *Nereis* and *Arbacia* agglutinating substances conclusively, for it was shown that *Nereis* egg-extracts strongly agglutinative for sperm of *Nereis* had no agglutinating effect on *Arbacia* sperm within the limits of attainable concentrations (several experiments). The *Arbacia* fluids are extremely toxic apparently to the *Nereis* sperm for the agglutinations were more solid than those caused by the iso-agglutinating substance. The absence of a reciprocal effect, that is, of *Nereis* extracts on *Arbacia* sperm, is therefore all the more striking.

The same difference may be shown by the reactions of sperm suspensions of both species to one another. The experiment was made in the following manner:

B. ARBACIA

The tests concerning the relation of the activity of spermatozoa of *Arbacia* to the chemical composition of the sea-water were not so extensive as in the case of *Nereis*. Moreover we do not have any definite aggregation reaction here to serve as a measure of activity. However, a sufficient number of tests were made to show that *Arbacia* is much less sensitive to variations in inorganic constituents than *Nereis*.

CO₂. To afford comparisons we may use three forms here, *Chaetopterus*, *Arbacia* and *Nereis*:

	100% CO ₂	40% CO ₂	20% CO ₂	10% CO ₂	5% CO ₂
<i>Chaetopterus</i>	paralyzed	few move slightly	many move	active	active
<i>Arbacia</i>	paralyzed	paralyzed	paralysed	paralyzed	paralyzed
<i>Nereis</i>	paralyzed	paralyzed	paralyzed	paralyzed	paralyzed
	2.5% CO ₂	1% CO ₂	0.5% CO ₂	NORMAL SEA-WATER	
<i>Chaetopterus</i>	active	active	active	active	
<i>Arbacia</i>	traces of movement	fairly active	active	maximum activity	
<i>Nereis</i>	paralyzed	paralyzed	fairly active	maximum activity (aggregation)	

Thus, while compared to *Chaetopterus*, *Arbacia*, is extremely sensitive to the presence of CO₂, compared to *Nereis* it is relatively insensitive.

To other acids, H₂SO₄, HCl, HNO₃, and CH₃COOH, *Arbacia* is also less sensitive than *Nereis*, exhibiting a fair degree of activity in N/1000 solutions in sea-water (compare table for *Nereis*, p. 526).

The sensitiveness to alkalis does not differ materially from that of *Nereis*. Agglutination of the spermatozoa is caused by N/2500 KOH, giving a very pretty precipitation picture in a vial. Such agglutinations are irreversible. Spermatozoa between the agglutinated masses may be in motion.

The relatively slight sensitiveness of *Arbacia* sperm to CO₂ is correlated with absence of any such striking aggregation effects

as are exhibited by suspensions of *Nereis* spermatozoa. But indications of the same kind of reaction may be seen under certain circumstances. Thus, a fresh suspension mounted beneath a raised cover will soon exhibit cloud effects due to differences in the density of aggregation, and this corresponds to the first stage of aggregation in *Nereis*. In the course of an hour or so all the spermatozoa retract from the edges into a central dense aggregation, and this is due, I believe, to the rising CO_2 tension towards the center. Reasons for this opinion are given under the head of the aggregation phenomena.

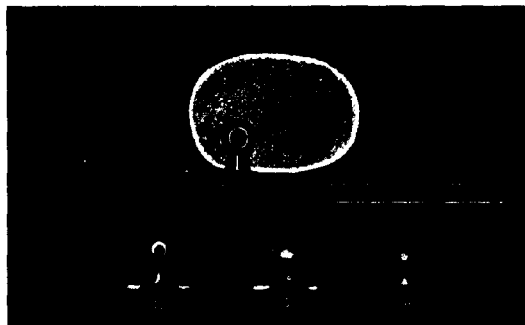


Fig. 4. Reaction of spermatozoa of *Nereis* to a drop of 1 per cent CO_2 sea-water; from an experiment of June 18, 1912. The original sperm suspension was made at 3.09; it aggregated clearly at 3.10 (fig. 1). It was then mixed up with a pipette and some drops mounted on a slide beneath a raised cover-slip as in 1. The drop of 1 per cent CO_2 sea-water was introduced at 3.12 $\frac{1}{2}$ (on left) and a drop of pure sea-water as control (drop to right). Figure 4, 1, shows the reaction at 3.13; 2, at 3.14; 3, at 3.14 $\frac{1}{2}$, and 4, at 3.16. In 4 the general suspension has aggregated. The final position of the reactive sperm is in the center of the introduced drop. No reaction takes place with reference to the drop of sea-water, which gradually becomes obliterated by inwandering of sperm. The figure shows also that the spermatozoa retract from the margin of the suspension.

III. AGGREGATION PHENOMENA

The spermatozoa of *Nereis* and *Arbacia* show very definite positive chemotaxis toward acids and egg-extracts of the same species, which may be demonstrated with striking clearness by the method first introduced by Jennings in studying the behavior of *Paramecium*. The method as applied to behavior of spermato-

zoa consists in mounting some drops of a sperm suspension beneath a long cover slip supported by glass rods, and injecting a drop of the fluid to be tested into the suspension. It then forms a clear drop within the milky suspension, and reaction at once begins at its borders. This method gives incomparably more delicate results than Pfeffer's method of using capillary glass tubes. The drop is confined above by the cover and below by the slide and diffusion takes place only at its margins; in this way a gradient is established. In the case of the capillary glass tubes diffusion is so slight from the open ends that no delicate reaction can be expected. So after a number of trials the capillary tube method was abandoned and the injected drop method was used exclusively.

A. NEREIS

1. Aggregation with reference to CO₂

As introduction, the reaction of a fresh suspension of the spermatozoa of Nereis to a 1 per cent dilution of sea-water saturated with CO₂ will first be described from a specific experiment:

June 26, 1912. A ripe male Nereis was placed in a dry watch crystal and snipped with scissors; two drops of the dry sperm were mixed in 10 cc. sea-water at 9.11½ A.M. and made a milky suspension which aggregated freely in thirty seconds. Some drops of this were then mounted beneath a cover slip supported by glass rods about 1 mm. in diameter. A drop of the 1 per cent CO₂ sea-water was injected at 9.13. In withdrawing the pipette a trail of the CO₂ sea-water is left extending to the margin. In a few seconds the following configuration developed (fig. 4-1). It consists essentially of a dense aggregation of very active spermatozoa in the form of a ring within the margin of the original drop, and a line extending from the drop to the edge where the pipette was introduced and withdrawn. In this case the ring is open below and a linear aggregation extends from the opening towards the margin of the suspension. The ring and the linear aggregation are separated from the general sperm suspension by a clear area devoid of spermatozoa 1.5 to 2 mm. in width. This area

belonged mainly to the original territory of the sperm suspension, and the ring owes its origin to migration of spermatozoa towards the drop. They do not, however, penetrate at first to the center of the drop, but their movements are arrested, hence the formation of the ring. The 'tail' of the ring is due to migration of spermatozoa to the trail of CO_2 sea-water left behind in withdrawing the pipette, leaving a clear zone marking the range of the effective stimulus. Control: No reaction is given to a drop of pure sea-water similarly introduced.

The migration of spermatozoa to the first formed ring continues for a short time; the ring thus grows broader and tends to close in the center (fig. 4-2 and 3). Shortly after the ring and tail aggregations have formed with reference to the introduced drop of CO_2 sea-water, the usual aggregations of the sperm, 1 to 2 mm. in diameter, form in the remainder of the suspension outside the drop evenly spaced throughout, if the sperm suspension is perfectly fresh (fig. 4-4). But if it is a little stale the general suspension remains homogeneous.

The detail of form of the ring and tail aggregations vary according to whether the introduced drop simply displaces a certain amount of the suspension, or is more or less mixed in the introduction; and this depends obviously on the size of the opening of the capillary pipette and the rate at which the drop is introduced. But the general form of the reaction is always the same.

The spermatozoa in the CO_2 aggregations are never in the least agglutinated and their behavior is in all essential respects the same as in the aggregations formed in any fresh suspension. I therefore early formed the hypothesis that the aggregation phenomenon is a chemotactic reaction to CO_2 produced by the spermatozoa themselves, and this hypothesis has been abundantly confirmed, as the series of experiments to be described will show.

The formation of the described configuration in a suspension of active spermatozoa with reference to an introduced drop is due to positive chemotaxis to the drop. If the clear margin be observed during the formation of the ring, the spermatozoa may be seen swimming across it to the ring head first. Under the low power of the microscope they appear to drift across it with a

dancing motion like motes in a sunbeam owing to their spiral path. If the external edge of the clear zone be carefully observed, the spermatozoa can be seen to detach themselves one by one from the general suspension and pass straight over to the ring. But only those freely suspended make the direct path; those in contact with the slide or cover continue their circus movements; the chemotactic stimulus seems unable to overcome the thigmotactic reaction.

The reaction is given most clearly and rapidly by a fresh sperm suspension, although one which has passed the aggregation stage still gives it; however, as the sperm suspension becomes stale the reaction becomes slower, and eventually ceases. Spermatozoa killed by gentle heat give no such reaction, thus excluding any purely physical diffusion effect as cause of the phenomenon.

In the case of this reaction in a somewhat stale non-aggregative suspension the movements of the spermatozoa on the outer margin of the ring are decidedly more vigorous than in the general suspension. This would appear to indicate that, at this place in the CO_2 gradient marked by the clear zone, the concentration of the CO_2 is stimulating rather than depressing; but when we consider that the CO_2 gradient must rise from the suspension across the clear zone to the ring, and that the relative inactivity of the sperm in the suspension is due, partially at least, to CO_2 the conclusion is not so clear. In any event, if we attribute a stimulating action to a given CO_2 concentration on such evidence, we must regard the depression of activity in the general suspension as due partly to other excreta.

The conditions established by the experiment may be represented diagrammatically as follows (fig. 5). The injected drop is represented by the continuous line circle and continuation, the general suspension by the shaded area. By diffusion from the injected drop a CO_2 gradient is established outwards, and this must extend into the drop a certain distance because the gradient is established by loss of CO_2 from the drop. The concentric broken lines represent the gradient, or at least that part of the gradient which is affective in the reaction. The thick open circle and the similar linear extension represent the aggregations of the sperma-

tozoa. At the same time there is of course diffusion of substances peculiar to the general suspension towards the introduced drop; but that conditions thus arising are ineffective is shown by the fact that no reaction is given to the introduced drop of pure sea-water. We may, therefore, leave this centripetal diffusion out of account. It should be remembered that 1 per cent CO_2 sea-water is the minimum paralyzing strength for *Nereis* sperm.

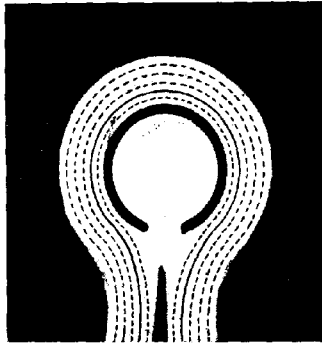


Fig. 5 Diagram of the reaction of a sperm-suspension of *Nereis* to an introduced drop of 1 per cent CO_2 sea-water; explanation in the text.

The diagram therefore shows, that migration of the spermatozoa proceeds up the gradient to, or near to, the point of paralysis of the spermatozoa; for in the case of the drop of 1 per cent CO_2 sea-water the ring forms well within the original margin of the drop. With higher and lower dilutions of CO_2 the width of the clear margin is practically the same.

The effects of greater and less CO_2 concentration than the 1 per cent used in the initial experiment are interesting. In general the use of a greater concentration involves a larger aggregation, and, of a less concentration a smaller aggregation. Thus if a drop of sea-water saturated with CO_2 be introduced into a suspension of fresh sperm beneath a raised cover slip a border of dead or paralyzed sperm forms at its margin, and shortly a clear zone forms external to it; the spermatozoa migrate in large

numbers across the clear margin to the ring and are speedily paralyzed by the diffusing CO_2 ; this process continues very rapidly and as a consequence the central aggregation expands, and may in time absorb all the spermatozoa of the suspension.

The same phenomenon in less pronounced form is exhibited by the reaction to a 1/10 dilution of the saturated CO_2 sea-water. Here we may give a definite experiment with measurements of growth of the aggregation: July 1, 1912. Into a fresh sperm suspension beneath a raised cover a drop each, (a) of 1 per cent, and (b) of 10 per cent CO_2 sea-water was injected some distance apart. Drop (a) measured 5 mm. in diameter and drop (b) 3 mm. immediately after injection at 2.28 P.M. The aggregations caused by these drops measured at 2.32: (a) 3 mm. (b) 5 mm. That is to say, the aggregations formed *inside* the drop in case of the weaker solution, and outside in the case of the stronger. At 2.35 (a) still measured 3 mm. and (b) now 6 mm. In a repetition of this experiment drops (a) and (b) each measured 3 mm. at 2.37. The aggregations caused by them measured at 2.40, (a) 2 mm., (b) 5 mm.; at 2.47, (a) 2 mm., (b) 10 mm.

It is clear from these observations that the spermatozoa are positively chemotactic in a CO_2 gradient where the tension is above a certain point, and that the aggregation caused by the more concentrated drop grows because the diffusion of CO_2 from the center forms a widening ring of the necessary concentration. To furnish a gradient the concentration must exceed the CO_2 tension in the general suspension, which is a function of the age of the sperm suspension and the activity of the spermatozoa in it, and on the other hand a limit is set to the differential which furnishes the reaction by the fact that a concentration of about 1 per cent of the saturated CO_2 sea-water paralyzes the spermatozoa. The gradient that furnishes the reaction must, therefore, operate within very narrow limits.

Greater dilutions of the CO_2 sea-water than 1/100 will act positively in the case of fresh sperm suspensions. The ring forms within the margin of the drop in the case of 1/200 dilution, but it remains narrow, and tends to break into bead-like aggregations, proving that the spermatozoa by their own activity have produced

a greater CO_2 concentration within the ring, which furnishes centers of aggregation positive to the $1/200$ concentration of the drop. Drops below this concentration or drops of pure sea-water furnish no reaction.

Summarizing; it would appear that the spermatozoa of *Nereis* follow a CO_2 gradient to the point of paralysis (about $1/100$ saturation). The clear zone outside an aggregation represents the effective CO_2 gradient in every case. The various forms of reaction to drops of different concentrations follow from this simple principle.

2. Interpretation of the aggregation reaction

We are now prepared for the interpretation of the aggregation phenomena exhibited by fresh sperm suspensions described on page 519. The spermatozoa as they come from the body cavity are absolutely quiescent; as soon as they are suspended in sea-water they become intensively active, and consequently produce CO_2 very rapidly. Any area of greater concentration of spermatozoa, by producing more CO_2 than other areas, becomes a center of attraction, and aggregations of the spermatozoa once begun are bound to proceed to the limit, because the closer the aggregation the greater the CO_2 production and consequently the greater the chemotactic stimulation. If aggregations once formed are broken up and the spermatozoa evenly suspended once more, the CO_2 tension in the suspension is greater than at first and is evenly distributed. Hence, in the first place the activity of the spermatozoa is reduced, and, in the second place, the differential of the gradient between that of the general suspension and the point of paralysis is greatly lessened. Therefore aggregation takes place more slowly and less completely than before; and, after a second and a third stirring up, the CO_2 tension in the entire suspension has become too great to permit of sufficient activity to react to the slight possible differential gradient.

It is obvious that such a reaction can take place only in the case of spermatozoa that exhibit extreme sensitiveness to CO_2 . The spermatozoa of *Nereis* possess by far the greatest sensitiveness to CO_2 of any studied, as we have already seen. No other

spermatozoa exhibit the aggregation reaction in any marked form so far as I know; Arbacia which comes next to Nereis in point of sensitiveness to CO_2 among the forms studied, shows, under certain conditions, a cloud-like formation similar to the initial stage of aggregation in Nereis. These will be referred to beyond.

It may perhaps be objected that the aggregation reaction in Nereis is not necessarily caused by CO_2 excretion, but possibly by some other substance produced by the spermatozoa. And it would be difficult to meet this objection in any absolutely conclusive way. But the following considerations render the conclusion extremely probable. In the first place it can be proved that the spermatozoa exhibit positive chemotaxis towards some substance that they themselves produce. Thus July 31, 1912, the following experiment was made:

With the dry sperm of one individual two suspensions were made at the same time (8.57 A.M.) namely: *a* one drop of sperm in 9 cc. sea-water, *b* two drops of sperm in 6 cc. sea-water; the activity of the sperm in both suspensions being the same *b* should produce any attractive substance in much greater amount than *a*. This was tested by making preparations of *a* and *b* on separate slides beneath raised covers. A drop of *b* was then injected into slide *a*, and a drop of *a* into slide *b* at 8.59. On slide *a* there was a very quick beautiful positive reaction to the introduced drop, that is a clear border formed about the drop owing to positive chemotaxis of the spermatozoa *a* to the drop of denser suspension *b*. On slide *b* not only was such positive reaction absent, but the drop of introduced sperm actually lost its spermatozoa and became clearer, owing to the positive chemotaxis now being away from the drop. The same results were obtained also by using two suspensions of equal density, one of which was older than the other. The fresher suspension reacted positively to the older suspension.

In the second place, it is of course certain that the spermatozoa becoming suddenly active in the sea-water must produce CO_2 ; and as we have seen that the spermatozoa of Nereis react even to a 1/200 dilution of a saturated solution of CO_2 in sea-water, if it can be proved that a standard suspension of spermatozoa produces an equivalent amount, the probability that CO_2 is the agent involved in the aggregation effect become very great. Experiments directed to this end showed that a 1/100 dilution of CO_2

lies near the limit of demonstrability both by color reaction tests and also by gas-burette estimation. A number of tests of sperm suspensions were made with acid color indicators. In the case of neutral red a dilute solution in sea-water has a decided orange tinge due to slight normal alkalinity of the sea-water. The same dilution made from a standard concentrated solution by the addition of a sperm suspension, shows a decided rose color without any trace of orange. The spermatozoa then aggregated in the vial used and the aggregations sank to the bottom, forming a bright red precipitate, and the supernatant fluid, now merely opalescent on account of the few sperms remaining in it, was faint rose. There is thus a decided acid reaction of the sperm suspension. Tests with azolitmin and tropaeolin 000 No. 1 also gave clear indications of acid. The sperm suspensions were tested within two minutes or less after their preparation; the liberation of the acid takes place therefore very suddenly. It is liberated only when the sperm become active, and the change of color is not given if the sperm remain inactive. It is therefore very probable that CO_2 is the acid revealed.

Finally a large number of tests for CO_2 were made of the air in closed flasks containing considerable quantities of active sperm suspensions of *Arbacia*. The details of these tests made with a gas burette need not be given. They extended over a week, using the sperm of *Arbacia* which could be obtained in larger quantities than *Nereis*. Although the determinations came very near the limits of experimental error, there could be no question as to the presence of CO_2 in quantities above that contained in the air or in normal sea-water.

In consideration of the facts (1) that sperm suspensions of *Nereis* produce a substance to which spermatozoa of *Nereis* react positively (2) that an acid is present in the suspensions (3) that the production of CO_2 by the suspensions can be demonstrated and (4) that spermatozoa of *Nereis* react positively to dilutions of CO_2 in sea-water which are barely detectable by color indicator, or gas burette, it can hardly be questioned that the aggregation reaction in *Nereis* is due to positive chemotaxis to CO_2 .

3. Reaction to other acids

The sperm of *Nereis* exhibits the same positive chemotaxis to other acids as to CO_2 . It is hardly necessary therefore to enter into details. Sulphuric, hydrochloric, nitric, and acetic acids were tested. They agree very closely with respect to the effective dilutions; N/1000 dilution of any of these acids is an effective chemotacticum agreeing quite closely in the degree of the effect produced with 1 per cent CO_2 ; N/2000 may cause slight ring formation in a drop introduced into a fresh sperm suspension, but, if the suspension has reached the non-aggregative stage, no reaction ensues, owing to the fact that the acid concentration in question furnishes no gradient.

Drops of stronger concentration cause a ring-shaped aggregation which continues to grow until diffusion eliminates the acid gradient. None of these acids cause the least sign of agglutination of the spermatozoa whatever their strength.

A drop of N/10 acid introduced within a sperm suspension beneath a raised cover kills all the spermatozoa in its immediate neighborhood, as the acid diffuses the zone of dead sperm increases but as the margin of the diffusing acid reaches a dilution that is no longer fatal it becomes marked by a clear border which is due to the migration of sperm to it, even though they are carried into a fatal concentration; and so the drop continues to grow so long as an acid gradient remains.

There is never the least sign of negative chemotaxis with respect to any concentration of any acid, nor indeed of any other agent tested. This being the case it is obvious that the aggregation of the spermatozoa can not be by any trial and error method of behavior, but must take place through orientation.

4. Behavior with reference to alkalis

The spermatozoa of *Nereis* do not exhibit any chemotactic reaction, positive or negative, to drops of KOH or NaOH injected into a suspension beneath a raised cover-slip. The drop remains empty at first, and spermatozoa that enter it by chance are agglutinated, so that in a short time the drop becomes filled

with agglutinated sperm masses. The alkalis were used in concentrations of N/2500 and N/5000.

Though no chemotatic reaction takes place, yet an interesting reaction may be procured by injecting a drop of N/5000 KOH into a sperm suspension made in 1/100 dilution of saturated CO_2 sea-water. The sperm in this are nearly or quite paralyzed, and will not of course react to an injected drop of 1/100 CO_2 . But if a drop of N/5000 KOH be injected into such a suspension beneath a raised cover glass, motility of the spermatozoa returns at a short distance from the margin of the introduced drop, due evidently to neutralization of the acid, and aggregations of the active sperm may form outlining the KOH drop a certain distance from the margin. This experiment furnishes at once an interesting demonstration of recovery from CO_2 narcosis, and of the nature of the contrast between the acid and alkali reaction of the spermatozoa.

5. Reactions to other substances

It lay entirely outside of the scope of this work to attempt an exhaustive analysis of the behavior of the spermatozoa with reference to chemical substances. The investigation was undertaken to analyze the relations of the behavior of spermatozoa to fertilization; and when the principle of chemotaxis was once demonstrated, and the relation of this chemotactic reaction to the very striking aggregation reaction, it seemed better to turn at once to the subject of the behavior of the spermatozoa towards eggs and egg-secretions of the same species. It may be remarked incidentally that tests with alcohol and ether gave negative results, that is, no evidence of positive or negative chemotaxis was found. In some cases there was slight indication of ring formation near the margin of an introduced drop of 5 per cent alcohol or ether in sea-water, which may indicate a stimulating effect of the spermatozoa at an optimum concentration, but which, in the absence of a clear margin external to the ring cannot indicate chemotaxis.

6. *Thermotaxis*

The spermatozoa of *Nereis* do not exhibit any positive response to drops of sea-water at higher temperatures. Into suspensions of spermatozoa in sea-water at 21°C. under raised cover-slips drops of sea-water at 44°C., 52°C., and 84°C. were injected successively. No ring formation occurred with reference to any of these drops. They did not fill up with sperm as rapidly as drops of sea-water at room temperature, but this is no doubt due to the paralysis that sets in, as previously noted above about 28°C. The only observable effect of the heated drops was that aggregations formed a little earlier in the general suspension near the margins of the introduced drop; and this is attributable to increased activity of the sperm owing to rise of temperature, hence increased CO₂ production in the zone of increased activity; and more rapid aggregation as a consequence.

7. *Thigmotaxis*

In contact with any solid object *Nereis* spermatozoa tend to carry out circus movements in an anti-clockwise direction, when fresh, but may soon come to rest. In any field of the microscope in a suspension beneath a cover glass one sees many of the spermatozoa in contact with the slide at rest, and many others carrying out the circus movements, while those that are freely suspended swim in spiral paths. The thigmotactic reaction then appears first to be the exaggeration of the rotation component of the ordinary locomotor movements, and second rest.

This reaction may of course come in conflict with the chemotactic reaction, as for instance in the clear margin external to the ring of spermatozoa produced in response to a drop of 1/100 CO₂ sea-water. Within this area all the freely suspended spermatozoa swim directly towards the ring, but those in contact with slide or cover-slip may continue their circus movements without any apparent directive effect from the CO₂ gradient. The thigmotactic stimulus appears thus to be more effective than the CO₂ gradient.

This thigmotactic reaction may be the starting point apparently of some of the aggregations formed in suspensions. Thus aggregations tend to form in the angle between the glass rod and the slide, which appear to owe their origin to the thigmotactic reaction; but when a considerable number of spermatozoa have accumulated in the angle their CO_2 excretion acts as a positive chemotactic stimulus on the sperm of the suspension, and a dense swarm soon forms along the rod, filling the angle and extending beyond it. Such a continuous swarm then tends to break into evenly spaced masses still in contact with the rod, owing to variations in CO_2 production. Thus thigmotaxis in this case is the initial cause of aggregations, which owe their subsequent growth to chemotaxis.

It may be that the thigmotactic reaction is a frequent cause of aggregations, particularly in suspensions that have produced considerable CO_2 when aggregations form only slowly and always in contact with the substratum. But in fresh suspensions this cannot be the case, for the aggregations first formed are freely suspended. In many cases aggregations may be seen to form with reference to firm strands or fibers of mucus in a suspension, and in such cases it appears probable that thigmotaxis and chemotaxis are combined.

8. Variations of reactions

So far as observed the behavior of sperm suspensions in sea-water may be quite fully explained by the forms of reaction described, and this brings the present section to a natural conclusion. But we may finally note certain variations of the reactions. The sperm suspensions were usually made, as stated, by mixing a drop or two of dry sperm with about 8 to 10 cc. of sea-water in a Syracuse watch crystal. This was done with a pipette, drawing in the suspension and squirting it out again until the sperm was evenly mixed. If this is done from one side, as is usually the case, a current is made across the dish to the opposite side and back along both sides, creating miniature whirlpools. Such currents of course come to rest in a few seconds, but when the aggregations become visible, ten to forty or more seconds later, they define very accurately the original currents. At first I thought natur-

ally of some rheotactic reaction. But on more careful examination and consideration the following explanation appears much more probable. Microscopic aggregations must begin to form while the water currents are still moving; they are then elongated by the friction in the direction of the current, and as they grow to macroscopic size the aggregations tend to preserve this form. The definition of the currents is due to the form of the aggregations rather than to their arrangement, and as they contract to spherical form the current-figures become less pronounced and very largely disappear.

Very interesting configurations may be produced in a sperm suspension of *Nereis* by dropping in dilute acids. In a few seconds quite complex wreath-like or festooned aggregations of spermatozoa appear at the site of the entering drop marking out accurately the distribution of the acid in the suspension. These of course vary with the strength of the acid, and the distance from which it is dropped.

If a few drops of a suspension of active *Nereis* sperm be mounted beneath a raised cover slip, it will be observed that the outer margin of the suspension for a width of 1 to 2 mm. soon becomes free from spermatozoa, thus tending to concentrate the suspension the same distance from the margin (fig. 4). This concentrated ring of the suspension then tends to form aggregations more rapidly than the more central parts. In the case of a suspension that is not perfectly fresh, aggregations may form only in this ring. The withdrawal of spermatozoa from the margin of the drop might at first thought be attributed to a negative chemotaxis towards oxygen. However, it is almost certainly not this, but a positive reaction towards the higher CO_2 tension of the interior of the drop. If a drop of sea-water saturated with oxygen be injected into a suspension beneath a raised cover, the spermatozoa avoid it in the same way that they do the free margin of the suspension.

The spermatozoa of *Nereis* make an acid indicator more delicate than any of the chemical dye indicators. In the course of some experiments I discovered quite accidentally, thus avoiding an awkward mistake, that the first few drops of water through any

available filter paper give a distinct acid test, using the spermatozoa of *Nereis* as indicator: A drop injected into a fresh sperm suspension invariably gave the ring formation with outer clear border, which is the characteristic and unmistakable acid reaction. This suggests the possible use of such cells as indicators in certain classes of experiments; some preliminary observations which I have made concerning CO_2 production of dividing eggs by this method are distinctly promising, though the results are complicated by the usual presence of other substances.

9. Chemotaxis to egg-secretions

The spermatozoa of *Nereis* exhibit positive chemotaxis to egg-secretions, which may be demonstrated in the same way as the positive chemotaxis to acids, but this subject, which is of course the most important part with reference to the fertilization problem, may be postponed to the next section dealing with agglutination phenomena, because it is always associated with agglutination.

B. ARBACIA

The reactions of Arbacia spermatozoa are essentially the same in principle as those of *Nereis*, but on account of the lesser sensitiveness of the spermatozoa, as noted in the section on activation phenomena, the reactions are much slower and less clearly defined. This may be illustrated by the notes on a single experiment: July 20, 1912. A fresh suspension of Arbacia sperm was made by mixing two drops of the dry sperm with 9 cc. of sea-water. The suspension appears milky, and the spermatozoa are decidedly active under the microscope. A portion was immediately mounted under a raised cover-slip and two drops of 5 per cent CO_2 sea-water were injected some distance apart. At first there appeared to be no reaction, as contrasted with *Nereis* in which ring formation is almost instantaneous under such circumstances. In two minutes the sites of the drops became more cloudy than the rest of the slide, and a faintly defined clear margin began to appear surrounding the drop. The picture gradually gained in definiteness until it became very clear. The central aggrega-

tions at first showed radiating strands of sperm visible to the naked eye, but then closed to form a solid drop, and this grew at its margins, preserving the clear external zone, until the clear margins of the two drops, at first several millimeters apart, ran together. Outside of the influence of the drops cloud formations appeared, corresponding to an early stage of the aggregations of *Nereis*.

The clear margin of the drops is not by any means so well defined as in *Nereis*. Moreover, the spermatozoa are so small that it is difficult to observe their behavior in the clear margin. However, there can be no doubt that the phenomenon is essentially the same as in *Nereis*, and that the aggregation in the drop, the appearance of the clear margin, and the growth of the aggregation are due to positive chemotaxis to CO_2 .

This reaction is given clearly only by a fresh sperm suspension. One ten minutes old does not give it, owing presumably to formation of CO_2 in the suspension.

A considerable number of tests were made. In some the reaction was much more rapid than in the experiment described. In one of these tests I injected drops of 20 per cent, 4 per cent and 1 per cent of the CO_2 sea-water near together. In the case of the 20 per cent a ring with external clear margin was formed in a few seconds. The ring did not close. The 4 per cent formed a ring which closed in its center. There was no reaction to the 1 per cent. The same suspension gave no reaction twenty-five minutes after it was mixed. In another case I got a faint reaction to 1 per cent CO_2 sea-water.

The fact of positive chemotaxis of *Arbacia* sperm to CO_2 dissolved in the sea-water was repeatedly demonstrated. In the case of a drop mounted beneath a raised cover it expresses itself by a gradual aggregation of the sperm towards the center, leaving the margins clear.

As is to be expected from the slower and less delicate reaction to CO_2 , as compared with *Nereis*, spermatozoa of *Arbacia* react also to other acids, but more slowly and not to so great dilutions. Thus in tests of N/10, N/50, N/250 and N/1000 H_2SO_4 , strong positive reactions were obtained for the first three, whereas only a faint shadowy reaction is given to N/1000. In the case of *Nereis*

it will be recalled that N/2000 gives a distinct reaction. Positive reactions were not secured with N/1000 HCl or HNO₃, but were with N/250. The ring formed in response to N/250 H₂SO₄ grows somewhat owing to diffusion of the acid; in the case of N/50 and N/10 H₂SO₄ the growth is much greater, in general in proportion to concentrations.

As in *Nereis* alkalis agglutinate, but cause no aggregation. KOH was tested in two ways: (1) addition of a small quantity of N/2000 KOH in sea-water to a vial of fresh sperm suspension caused macroscopic agglutinations which are irreversible. (2) a drop of N/2000 KOH injected into a suspension beneath a raised cover fills in a short time with sperms that agglutinate; but there is no chemotaxis, and in a short while the drop is left to one side by aggregation of the sperm away from it, owing to rise of CO₂ tension elsewhere.

Reaction to egg-secretions

As contrasted with the slowness of reaction of *Arbacia* spermatozoa to acids, the reaction to egg-secretions is instantaneous and clear cut. There is a most pronounced positive chemotaxis, as tested with drops even of very weak egg-extract injected into a sperm suspension mounted beneath a raised cover-slip; but this is always associated with agglutination, and is, therefore, best considered under that head.

IV. AGGLUTINATION PHENOMENA AND REACTIONS OF SPERMATOZOA TO EGG-SECRETIONS

1. INTRODUCTION

Following the demonstration of definite chemotactic behavior of spermatozoa of *Nereis* and *Arbacia* the question naturally arises what relation, if any, has this form of behavior to the union of egg and spermatozoon in fertilization. As is well known, the mere observation of the interaction of the sexual elements in fertilization led long ago to the theory that the egg attracts the spermatozoon to itself by chemotaxis, and fusion results from active penetration of the ovum by the spermatozoon. But in recent years there has been a tendency to deny both of these

principles as factors in the union. Chemotaxis has fallen into disrepute; and the theory that the spermatozoon bores into the egg has been rejected by several observers.

If chemotaxis is concerned in the union of ovum and spermatozoon the medium in which fertilization operates must contain the substance concerned. In the case of the eggs of *Nereis* and *Arbacia*, therefore, the hypothetical substance which attracts the spermatozoa must exist in sea-water which has been in contact with fertilizable eggs; and it must be possible to obtain a sufficient concentration of the substance in question in sea-water to demonstrate its presence by reaction of the spermatozoa, because, *ex hyp.*, the substance exists in effective amounts in the sea-water surrounding the eggs. If it were impossible to demonstrate the presence of an agent to which spermatozoa of the same species are positively chemotactic by such means the theory of chemotaxis would have to be abandoned. However, the presence of such a substance is readily demonstrated both in *Nereis* and *Arbacia*.

In the second place, if the union of the ovum and spermatozoon after they have come in contact operates not mechanically, but through some bio-chemical reaction between spermatozoon and ovum, the sea-water in which eggs have been standing should contain a substance also capable of reaction with the sperm, which should be an efficient indicator for it.

I was guided by some such ideas as these in the series of experiments which follow, and which showed at the very first trials that sea-water which has stood with fertilizable eggs of *Nereis* or *Arbacia* contains a substance to which the spermatozoa of the same species are positively chemotactic, and also a substance which agglutinates the spermatozoa of its own species. It may be that one substance is concerned in both reactions, but it is more probable that two are present. It is perhaps worth emphasizing here, for this is the fact that struck me at the start, that the sea-water which has stood over eggs³ combines both the effects of

³ To avoid the frequent repetition of such a circumlocution we may call sea-water, which has contained eggs and is charged with their emanation, egg sea-water; and the concentration of the substance in the sea-water may be expressed by writing the relative bulks of eggs and sea-water as a fraction. Thus 'egg sea-water 1/3' would indicate that the bulk of eggs was one-third the volume of the sea-water. Time is also a factor in the concentration, of course.

an acid (aggregation) and also an alkali (agglutination) on the spermatozoa. The comparison may, of course, be superficial, but it serves at least to emphasize the double action of the egg-secretion.

As contrasted with the difference in rapidity and delicacy of reaction between the spermatozoa of *Nereis* and those of *Arbacia* to inorganic substances, we may note in advance that the reactions to the egg extractives are as rapid and clear in the one case as in the other, and are entirely similar in principle, though there are certain secondary differences that will be noted in the proper place.

2. INITIAL EXPERIMENT

We may begin by describing the reactions to be observed in the case of an *Arbacia* sperm-suspension freshly made and mounted beneath a raised cover-slip, into which a drop of *Arbacia* egg sea-water 1/10 to 1/20 about half-an-hour-old is injected. The naked eye observation shows almost instantaneous formation of a ring at the margin of the drop, with simultaneous formation of a clear external zone about 1.5 to 2 mm. wide; the ring then breaks up into small agglutinated masses and so becomes beaded. The trail of substance left in withdrawing the pipette extends to the margin of the cover-slip. It also is a center of attraction and the ring is therefore prolonged by a chain of agglutinated masses to the margin.

One can observe the details of the reaction best under the microscope, using a low power, by bringing the point of the pipette into the field of the microscope and blowing in the drop with the aid of a flexible rubber tube held in the mouth, while looking through the microscope. The reaction takes place so rapidly that it requires repeated observations to observe all the details. In the first second the spermatozoa are aroused to intense activity and form small agglutinated masses within the drop; these then appear actually to 'rush' together (to use the language of my note book) to form larger agglutinations for a period of three to five seconds, after which no more fusion of masses takes place. The agglutinated masses thus range from relatively large to relatively small. While this has been going on in the interior of the drop, a ring

has formed at the margin, and a clear zone arises external to it. The ring is at first continuous, but it ruptures in numerous places in two or three seconds and each segment contracts quickly to an agglutination mass.

The agglutinated masses in the interior of the drop are smaller than in the ring, owing to the relatively low concentration of the sperm suspension within the drop, and they break up very quickly while the sperm is still extremely active. The movements of the spermatozoa then gradually slacken; in a few minutes the larger and more firmly agglutinated masses of the ring also begin to break up and in ten or fifteen minutes all are resolved.

This preliminary observation demonstrates a three-fold action of the egg-extractive: (1) it activates the spermatozoa; (2) it aggregates them through positive chemotaxis; (3) it agglutinates them. The phase of increase of activity lasts only a short time, a minute or two at the most, after which movements of the sperm slacken and become less than the control, or cease entirely. Positive chemotaxis (aggregation) is shown by formation of a clear zone external to the marginal ring. This is always a sign of chemotaxis, as we have seen in the preceding section.

The agglutination phenomenon is fundamentally different from the aggregation; in the latter the spermatozoa are merely loosely associated, and slight agitation is sufficient to scatter them. In the agglutinated masses the spermatozoa are stuck together and are not separated by shaking. In the case of *Nereis* where the agglutination is firmer than in *Arbacia* the masses may be broken up into smaller masses by needles, or preserved *en masse* in killing fluids. The breaking up of the ring into separate masses is a characteristic agglutination effect; the rings formed in response to an acid do not break up unless the acid is very weak (see p. 537). Finally an agglutinative substance produces its effect when shaken up and evenly distributed in a vial of sperm suspension, but an aggregative substance cannot of course exert a chemotactic effect in the absence of a gradient.

The same experiment succeeds well with *Nereis*. The eggs of this form give off a substance (or substances) into the sea-water, which causes aggregation and agglutination of the spermatozoa

when a drop of sea-water so charged is injected into a fresh sperm suspension beneath a raised cover slip. The activation is not so pronounced in this case as in *Arbacia*. The aggregation phenomenon is the same. The agglutinations are substantially permanent in *Nereis*; the spermatozoa stick together much more firmly.

In what follows we may leave the activation and aggregation phenomena out of account for the most part and confine ourselves to the problems of agglutination. The substance which causes agglutination of the spermatozoa we shall call the sperm agglutinin. The agglutination may be shown very strikingly in a vial of fresh sperm suspension. In the case of *Arbacia* the addition of two or three drops of egg sea-water $1/4$, which has stood half-an-hour, to about 2 cc. of a fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half-an-hour or more.

The degree of agglutination is of course dependent on the density of the suspension. This is shown by the following experiment: Ovaries and eggs of *Arbacia* were cut up in about four times their own bulk of sea-water and allowed to settle. Two cubic centimeters of the supernatant fluid was put in each of three vials. To one was added 3 drops of a fresh milky sperm suspension, to the next 12 drops of the same, to the third 36 drops. No visible agglutinations formed in the first; in the second agglutinations became visible to the naked eye almost immediately, in the third agglutinations were larger, more numerous, and apparently more solid.

3. OVA ALONE PRODUCE THE AGGLUTINATING SUBSTANCE

The eggs of both forms thus produce an agglutinin in the sea-water. The next question is whether the agglutinin is specifically an egg-product. A considerable number of experiments prove that this is the case. The large body cavity of *Arbacia* is filled with abundant coelomic fluid and this may be supposed to

contain substances from various tissues. But it invariably proved perfectly neutral to spermatozoa of *Arbacia*, even when taken from females with large ovaries, showing that the substance concerned in agglutination does not escape from the ovaries of the intact animal, or, if it does, that it is promptly destroyed. Nor was it possible to extract a sperm agglutinating substance for *Arbacia* by extracting the intestine in sea- or fresh-water.⁴ These experiments were repeated a sufficient number of times to be conclusive.

As illustrations: (1) Intestine extractives. August 31, 1912. The intestines of several *Arbacia* were cut up in about twice their bulk of distilled water, and were allowed to stand in it about an hour. The strongly amber-colored fluid was filtered off and rendered isotonic with the sea-water by addition of concentrated sea-water (four parts of the latter to six of the intestine extract). This fluid causes no agglutination in sperm suspensions. A similar extract of the ovaries caused immediate large dense agglutination masses. (2) Coelomic fluid: As is well known, the coelomic fluid of *Arbacia* contains large numbers of densely pigmented corpuscles. Outside the body the fluid quickly forms a loose clot which includes many of the corpuscles. The others can be separated from the remaining serum by centrifuging. The corpuscle-free serum was sometimes used, sometimes simply the clot-free serum. Repeated tests were made both by injecting drops into fresh sperm suspensions beneath raised cover glasses, and also by mixing with fresh sperm suspension in vials. Whether the coelomic fluid came from males or females it proved invariably negative, except for the faintest sort of agglutination reaction in one or two cases only, which may have indicated some individual differences. It is interesting in view of these facts that outside of the body the coelomic fluid becomes heavily charged with the sperm agglutinin if eggs are placed in it. It must be supposed therefore that the ovarian membrane is impermeable to the agglutinating substance in the intact animal.

⁴ The experiments this year simply opened up the problems, and it was impossible to make any quantitative tests or adequate chemical examination. For the purpose of the biological problem of the behavior of the spermatozoa with reference to the eggs the question of immediate importance was the behavior with reference to egg-extractives or secretions in the sea-water tested. Stronger agglutinating solutions were made by increasing the quantity of eggs with reference to the sea-water, or by crushing the eggs in sea-water. Distilled water was shown to extract more agglutinin from a given bulk of eggs than sea-water; and the coelomic fluid of *Arbacia* also proved to be a better medium for extracting agglutinin from the eggs than sea-water. It would be possible of course to establish quantitative values for all of these relations, and this problem should receive attention. The problems of solubility of the agglutinin in various media, and other chemical questions, also present themselves.

In the case of *Nereis* it was not possible to reach such conclusive results, because the sexually mature female is practically a bag of eggs, and one cannot obtain other organs for testing. I cut up five females that had shed their eggs in 10 cc. of sea-water. In spite of efforts to get rid of all eggs, a considerable number were in the water. For control I used the eggs of two females in 100 cc. sea-water. On test in half-an-hour the fluid from above the eggs was found to be about ten times as agglutinative as the fluid from the bodies of the spent females. So that it is certain that other tissues do not produce much sperm agglutinin and it is probable that they do not produce any. The small amount present could be accounted for by the few eggs included, and perhaps by egg secretions absorbed by the tissues.

4. FIXATION OF THE AGGLUTININ BY SPERMATOZOA

The next question was whether the agglutination reaction as described has the usual characters of a chemical reaction? The general result is (1) that an agglutinated sperm suspension in which reversal has occurred is not capable of re-agglutination by addition of more of the agglutinating substance and (2) that the agglutinating substance disappears from an agglutinated suspension if not present originally in excess.

As regards the first point, the earlier experiments were concerned entirely with the form and conditions of the reaction, and the agglutinating substance was always used, as later results showed, in excess. It was not possible to get a repetition of the agglutination reaction under these circumstances. But one can get a repetition of the reaction in a sperm suspension by addition of successive small amounts of the agglutinating substance, until the reaction is complete, as the following experiment shows:

September 4, 1912. *Arbacia*. 2 cc. of a creamy active sperm suspension was agglutinated with 5 drops of an egg-extract prepared as follows: The ovaries of three females were cut up in about three times their volume of distilled water and allowed to stand about thirty minutes. Then the water was filtered off and made isotonic with sea-water by the addition of concentrated sea-water (proportions of 58 to 42 parts). This made a very strong agglutinating extract. After reversal of the agglutination described above the agglutination was repeated by addition of a drop of

another egg-extract. This time the agglutination was complete for it could not be repeated a third time. Other sperm suspensions gave similar results.

The result might be interpreted as a purely biological reaction, that is to say in terms of stimulation, were it not for the fact that the agglutinating substance disappears from an agglutinated sperm suspension, as shown by the following experiment:

September 12, 1912. *Arbacia*. Nine parts of a thick active sperm suspension was agglutinated by one part egg-extract. The agglutination produced was so strong that the fluid between the white masses appeared clear to the eye. In three or four minutes reversal of the agglutinations had begun. The agglutinated sperm suspension was then centrifuged until practically all the sperm was precipitated. The supernatant fluid was tested and agglutinin was shown to be absent. As control, a dilution of one part of the same egg-extract with 9 parts of sea-water was tested with the same sperm and proved to be strongly agglutinative. Three tests were made with each with uniform results.

There can be no doubt, as the result of this and other observations also, that the spermatozoa fix in some way the agglutinating substance, and it will be simplest to assume as a working hypothesis that the fixation is due to chemical union. I have not yet had the opportunity to ascertain if the agglutinin could be regained from the sperm precipitated in the centrifuge.

5. NATURE OF THE EFFECT ON THE SPERM

We have noted four effects of the egg-extracts on sperm of the same species, namely: (1) Stimulation of intense activity, which is of brief duration. This is more marked in the case of the sperm of *Arbacia*, than in the case of the naturally extremely active sperm of *Nereis*; (2) An orienting effect expressed in positive chemotaxis; (3) An agglutinating action; (4) Following these effects more or less complete paralysis of the sperm.

To what kind of change in the individual spermatozoa is the agglutination reaction due? We may note in the first place that the agglutination is between the heads of the spermatozoa, and that the tails are apparently unaffected, at least at first; it is only in later stages of the action of the agglutinating substance that the locomotor function is injured. The adhesion of the heads

demonstrates some change in the membrane that renders them sticky. The cells are so minute that it is difficult to observe any microscopical change in the case of *Arbacia*; in *Nereis* the spermatozoa are larger, and it can be seen that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one, indicating a great increase in permeability. The spermatozoa which have undergone this change are usually motionless, and, when not fused with one another, appear to be glued to the slide or cover slip, never freely suspended.

Agglutination in itself is in no sense a specific reaction, but one that may be expected to accompany certain superficial changes of the spermatozoa, however caused, under conditions that bring the spermatozoa into contact. It occurs, to a limited extent, spontaneously in sperm suspensions that have stood for some time. It is particularly noticeable in *Nereis* under the following conditions: A fresh sperm suspension is allowed to aggregate on a slide beneath a raised cover slip and the aggregations remain undisturbed. In the course of ten or fifteen minutes small agglutinated masses may form around the margins of the aggregations or beneath the aggregations in contact with the slide. There may be twenty to fifty or more such masses associated with a single aggregation, and they are quite similar in their general appearance, to those produced suddenly by the agglutinin of the egg, though much smaller, on the average.

It is not probable that the agglutination is in any real sense toxic or cytolytic. It is true that the agglutinin inhibits movement after a few minutes, and it certainly lessens the fertilizing power of the sperm. But if an agglutinated mass of spermatozoa of *Nereis* be crushed under the microscope many of those liberated are active. Moreover, if a small quantity of an agglutinated suspension of spermatozoa be added to a relatively large quantity of sea-water fertilizing power is partially regained. This might be either because some of the spermatozoa of the agglutinated suspension had escaped combination with the agglutinin and were alone concerned in the actual fertilization, or because of recovery

from the agglutination effect. It is very difficult to form a definite opinion as to the real nature of the agglutination effect.

6. THERMO-RESISTANCE OF THE AGGLUTININ

The agglutinating agent is slowly destroyed at 95°C.:

August 28. *Arbacia*: (1) Ovaries and eggs of *Arbacia* were cut up in three times their bulk of sea-water, and let stand about an hour. The supernatant fluid is strongly agglutinative on *Arbacia* sperm suspensions. (a) Part of it was now taken and boiled about thirty seconds, and cooled. On test it proved as agglutinative as before. (b) Some more was then boiled five minutes and cooled. Its agglutinative power was apparently undiminished. (c) Another egg-extract similar to the first was then boiled and put in a beaker of boiling water for thirty minutes; the temperature stood about 95° during this process. On test its agglutinative power was shown to be greatly diminished. (d) In a fourth test some egg-extract was kept at 95° for sixty-six minutes. It still exhibited some agglutinative power, which, however, was very slight as compared with the control. The heated egg-extract exhibited a considerable change of color from the yellowish red of the control to a much brighter red.

Simultaneously with the loss in agglutinating power, it appeared also to gain in aggregating power: Drops of the heated and the unheated egg-extract were injected into the same sperm-extract beneath a raised cover. The drop of the heated expanded its sphere of influence shown by immigration of the sperm about twice as rapidly as the unheated; this was tested several times; so that it would appear that the aggregative and agglutinative agents are probably distinct, and that the agglutinin inhibits aggregation to a considerable extent.

Nereis: The eggs of three females were inseminated in 9 cc. sea-water; the supernatant fluid has a slight amberish-green color, and is strongly agglutinative on *Nereis* sperm. (a) After keeping at 95° C. for ten minutes its agglutinative power was much reduced. (b) After twenty-two minutes at 95°C. the agglutinin was entirely destroyed. The color was entirely destroyed also.

Thus the agglutinating substances, whatever they may be, are either volatile being gradually driven off by heating, or they are slowly coagulated or disintegrated chemically by a temperature of 95°C. The agglutinin of *Nereis* is either more volatile or more labile than that of *Arbacia*. It is impossible to say definitely to what class of chemical substances these agglutinating

substances belong. It is, however, extremely improbable that they possess a degree of chemical simplicity sufficient to allow of volatilizing; it is more probable that they undergo slow chemical disintegration at the temperature employed. The thermostability of these sperm isoagglutinins is relatively very high, and this perhaps makes it doubtful whether they can belong to the same class of substances as the haem-agglutinins of vertebrate blood sera. This matter must therefore remain undecided, and it should be understood that the term agglutinin is used in the present paper in a purely descriptive sense.⁵

7. FERTILIZING POWER OF AGGLUTINATED SPERM

The powerful effect of the egg-extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power. The following experiments illustrate this:

1. *Arbacia*. The egg-extract used was made by cutting up the ripe ovaries in about three times their bulk of distilled water; in half-an-hour the water was filtered off and was then made isotonic with sea-water by the addition of 42 parts of condensed sea-water to 58 of the egg-extract. Five small watch crystals in a series contained (1) 8 drops of the egg-extract (2) 4 drops egg-extract, + 4 drops sea-water (3) 2 drops egg-extract, + 6 drops sea-water, (4) 1 drop egg-extract, + 7 drops sea-water (5) 8 drops sea-water. To each of these 3 drops of opalescent sperm suspension was added; and after twelve minutes a drop of a suspension of fresh eggs was added to each. We thus had the same quantity of eggs in sperm suspensions of the same density, but in graded amounts of egg-extract. The sperm suspensions were so dense that in the control (no. 5) the jelly became packed with sperm, forming dense halos around the eggs. In (4) a very few (about 1 per cent) had slight halos of sperm in the outer layer of the jelly, but in (1), (2) and (3) the paralysis of the sperm was so complete that they did not enter the jelly of the eggs at all. Ninety-seven minutes later none of the eggs in (1), (2), (3) or (4) had segmented; whereas at least 5 per cent of the control were now in the two-celled stage. The lot of eggs was rather poor in this case, but fertilization was confined entirely to the control.

If the experiment be made in another way some recovery of the spermatozoa from their state of paralysis may be observed. Thus: An

⁵ It should be borne in mind that but little is known concerning lysins or agglutinins of invertebrates. It is perhaps not to be expected that they should exhibit the same degree of thermolability as those of vertebrates.

active sperm suspension was divided in two parts, and one part was agglutinated by the addition of about 40 per cent of its own volume of the egg-extract described above, to the other an equal amount of sea-water was added. The first was strongly agglutinated; after reversal both suspensions were stirred up, and beginning thirteen minutes after agglutination a series of fertilizations were carried out by adding one drop of the agglutinated sperm suspension to a measured quantity of eggs in about 9 cc. of sea-water at 10 minute intervals. Each fertilization had a control of the same quantity of eggs fertilized with one drop of the control sperm. The consistent result was that about 16 per cent of the eggs fertilized with the agglutinated sperm segmented and at least 33 per cent of the control. The non-agglutinated spermatozoa are about twice as effective as the agglutinated. But a considerable degree of recovery of some spermatozoa of the agglutinated suspension is shown.

2. *Nereis*. Experiments with *Nereis* did not give such a marked reduction of the fertilizing power of agglutinated sperm as in *Arbacia*. There was, however, a marked delay in the formation of jelly when agglutinated sperm was used as compared with normal sperm.

It is somewhat difficult to make a satisfactory interpretation of the effect of agglutination on fertilizing power. On the one hand we may suppose that a certain proportion of spermatozoa resist the agglutination effect, and are alone concerned in any fertilizing power of an agglutinated suspension; on the other hand it might be supposed that the agglutinating effect does not modify fertilizing power except as it decreases the motility of the spermatozoa or that the effect is reversible under the condition of the experiments. Either assumption would be consistent with the facts.

8. CONDITIONS OF FORMATION OF THE AGGLUTININ BY THE EGGS

The conditions of excretion of the agglutinating substance by the eggs into the sea-water is quite different in the two forms. In *Arbacia* the agglutinin is excreted continuously by unfertilized eggs in such amounts that I have not succeeded even by repeated washings in removing it all. Fertilization does not appear to increase or decrease the quantity. In *Nereis*, on the other hand, unfertilized eggs secrete but little of it, and one or two washings in sea-water will completely remove it so that the eggs secrete no more in detectable quantities; but at the moment of fertilization, on the other hand, it is poured forth in advance of the jelly in

large quantities and the eggs then appear to have disposed of their entire store, for washed fertilized eggs no longer produce it. The conditions are important in their bearing upon the question of permeability of the egg-membrane with reference to fertilization. The spermatozoa are very efficient indicators of substances leaving the egg. In the case of *Nereis* it can be shown that there is a sudden increase of permeability at the moment of fertilization, but in *Arbacia* such evidence is lacking.

The conditions in *Arbacia* may be shown by the following experiment: Eggs were washed free from all fragments of ovary and placed in about 20 times their own bulk of sea-water and divided in three lots, *a*, *b* and *c*. The sea-water over them agglutinates *Arbacia* sperm instantaneously. This test was made at 9.30 A.M. The supernatant fluid was then removed and the eggs washed in 20 times their bulk of sea-water. At 9.53 the supernatant water was tested and found agglutinative. 9.58, *a* and *b* were again washed. 10.04, supernatant fluid again agglutinative. 10.05, lot *a* was fertilized with 2 drops of sperm. 10.10, *a* and *b* tested again; both agglutinate. 10.16, *a* and *b* washed again. 10.23, supernatant fluid of both agglutinates. 10.30, *a* and *b* washed again. 10.36, both agglutinate sperm; *b* is rather more effective. 10.40, washed *a* and *b* again. 10.45, both agglutinate; *b* more effective. 10.51, washed *a* and *b* again. 11.00, both agglutinative; *a* more than *b*. 11.08 to 11.20, other tests of *a* and *b* show *a* somewhat more effective.

The experiment shows both fertilized and unfertilized eggs of *Arbacia* to be constantly secreting a substance into the sea-water which agglutinates the spermatozoa. The substance must be effective in very minute quantities for the amount of water used in each of the eight washings was at least ten times the bulk of the eggs, yet as soon as the eggs had settled to the bottom of the vials used the supernatant fluid contained the agglutinin in appreciable amounts. How long this process keeps up in *Arbacia* I cannot say; and too much reliance cannot be placed on the result for the fertilized eggs because a large proportion of the eggs failed to fertilize or at least to segment.

The conditions in *Nereis* are quite different; the experiments showed that shortly after the eggs are taken they charge at least ten times their bulk of sea-water with an easily detectable amount of sperm agglutinin. But if the eggs are washed once usually no more agglutinin can be demonstrated. If now the eggs are stirred

up in the vial, fertilized and allowed to settle, the supernatant fluid is very powerfully agglutinative. The experiment was repeated a sufficient number of times to make certain of this result.

A number of tests were also made with the object of determining if the eggs of *Nereis* continued to produce agglutinin after fertilization. These showed that the eggs cease very quickly their production of agglutinating substance. None could be detected during the maturation period, but apparently there is a second production about the time of the first cleavage. On this point I wish to be understood to speak with reserve. The swelling of the jelly secreted at fertilization makes the eggs very bulky, and the jelly itself takes up any egg secretion, so that there are considerable technical difficulties in making satisfactory tests.

The fact that stands out perfectly plainly in the case of *Nereis* is the sudden increase in secretion of agglutinin into the seawater just after insemination, followed by cessation of its production. The spermatozoa give absolutely positive tests. As will be shown later, the observations on the normal fertilization are in complete harmony with this.

The difference between *Nereis* and *Arbacia* in these respects is thus sharply marked. However, it should be remembered that the unfertilized eggs of *Arbacia* have formed both polar bodies, whereas those of *Nereis* are in the stage of the germinal vesicle. It may be that eggs of *Arbacia* in the germinal vesicle stage are relatively impermeable in the same sense as those of *Nereis*.

9. HETERO-AGGLUTINATION AND THE QUESTION OF SPECIFICITY: REACTIONS BETWEEN *NEREIS* AND *ARBACIA*

The demonstration of intraspecific sperm-agglutinating substances derived only from the ova having been made, the question arose whether these substances were essentially the same in both species, or different. If the same, the egg-extract of each should agglutinate the sperm of the other. A number of tests were therefore made which demonstrated conclusively that the substances are decidedly different with reference to their cross-agglu-

The fact that the egg secretions of Arbacia cause agglutination of Nereis sperm as well as Arbacia sperm seems at first sight to indicate the iso-agglutinating substance of Arbacia is not specific. But the fact that other Arbacia fluids likewise agglutinate the sperm of Nereis, but not those of Arbacia, raises the question whether the egg-secretion does not contain in addition to the iso-agglutinating substance, also another which is agglutinative for Nereis sperm like the substance in the coelomic fluid, but not for its own sperm.

If there are two substances present in the egg secretion it ought to be possible to separate them by various means. They might exhibit different heat-lability, so that one might be destroyed at a temperature that would leave the other still active. Or if they have different affinities it might be possible to fix the Nereis agglutinating substance by Nereis sperm, leaving the iso-agglutinating substance intact. Neither of these experiments could be performed this year, owing to the disappearance of the necessary material.

However, it was possible to show in another way that the Nereis-agglutinating substance of Arbacia egg-extract is distinct from the iso-agglutinating substance: An egg-extract of Arbacia seventeen days old was found to have entirely lost its power of agglutinating Nereis spermatozoa, while it retained undiminished its power of agglutinating Arbacia spermatozoa. Originally it agglutinated both kinds of spermatozoa. Now the change that took place in the egg-extract on standing is not a mere weakening of action as might be supposed, because the iso-agglutinating action was noted as undiminished, whereas the hetero-agglutinating action was entirely lost. The only possible conclusion, therefore, is that the egg-extract contained two agglutinating substances at least, namely: An iso-agglutinin and a hetero-agglutinin, and that the latter is relatively labile, the former relatively stable. Unfortunately the experiment could not be repeated on account of the total disappearance of the material.

fact, an almost necessary conception on the general principles of immunity phenomena that the sperm should so act. The question would be, of course, whether there is a connection between any antibodies so formed and the sperm iso-agglutinins produced by the next generation of ova.

The experiment in detail was as follows:

September 15, 1912. One small male *Nereis* available. Its sperm is aggregative and very active. The *Arbacia* egg-extract was of August 29 and was made by cutting up ovaries and eggs of *Arbacia* in four times their volume of sea-water. After settling of the eggs the supernatant fluid was poured off, and had been kept in a stoppered vial since.

1. A drop of the *Nereis* sperm and a drop of the egg-extract were placed side by side on a slide and connected; the sperm diffused into the egg-extract and swam around in it; no agglutination.

2. A raised cover mount was made of the *Nereis* sperm and a drop of the egg-extract injected. The sperm entering the drop swam around, and were not agglutinated.

Controls: 1. The sperm of *Nereis* was agglutinated immediately by extract of *Nereis* eggs kept since September 8.

2. The egg-extract of *Arbacia* agglutinated *Arbacia* spermatozoa with no apparent diminution in the strength of the reaction.

Conclusion: The nonspecific agglutinating substance has been destroyed by the chemical changes in the extract in the course of seventeen days; but the iso-agglutinating sperm substance still remains.

This experiment does not demonstrate that the sperm iso-agglutinin of *Arbacia* egg-extract is specific, but merely that it is without effect on the *Nereis* sperm, just as the iso-agglutinin of *Nereis* eggs is without effect on *Arbacia* sperm. It is of course still possible that the iso-agglutinating substances might have an agglutinating effect on some other varieties of sperm, and a crucial test of specificity must await the securing of new material.

However, it seems to me that the probabilities in the case lie strongly on the side of specificity of these sperm iso-agglutinating substances. Quite apart from the value of the evidence already adduced, we must consider the general fact that ova and spermatozoa of the same species do behave in a specific way with reference to one another in the process of fertilization. This must have some chemical basis and on the chemical side the only reactions that exhibit a corresponding degree of specificity are those between antigens and anti-bodies in the field of immunity. We have two parallel instances, therefore, and the slight evidence which I have so far been able to bring forward in favor of the specificity of the sperm iso-agglutinins of ova gains immensely in weight by its association with the universal principle of specificity in fertilization, and the known class of specificities in agglutination reactions.

The agglutination of spermatozoa is, of course, in itself of no significance for the problem of fertilization; the spermatozoa unite in fertilization with the egg, not with one another. The agglutination reaction is, however, an indicator of an important change in the spermatozoon in the presence of egg secretions, and therefore evidence of a change that any spermatozoon must undergo when it comes in contact with the egg. The adhesive property that the sperm develops under these circumstances may be an important factor in binding the sperm to the egg until it can be incorporated. But, if the reaction be specific, it is much more than this; it is evidence of an intimate chemical combination of sperm and egg constituents which begins at the very moment of union.

Von Dungern's experiments ('02) are the only ones, so far as I know, in which the production of sperm agglutinins by ova was investigated, and he discovered only hetero-agglutinins, no iso-agglutinins. He did, indeed, describe the loss of motility of spermatozoa in egg-extracts of the same species, but he entirely missed the phenomenon of agglutination and its reversal. He reveals the reason for this failure by his remark that he always examined for the effect of the 'egg-poison' about half-an-hour after its addition to the sperm; but the phenomenon of agglutination and its reversal are completed in about five minutes.

Von Dungern also made experiments on the production of immune sera by injection of ova and spermatozoa separately into rabbits, and found that both caused the production of a sperm agglutinin in the rabbit's serum. From this he concludes that both kinds of reproductive elements possess chemically identical complexes of molecules in the protoplasm. While this may be admitted as at least a very probable conclusion, his farther conclusion that fertilization does not depend upon any specific antagonism between ovum and sperm, but is conditioned by the similarity of their protoplasms, is not well founded, for the egg is a very complicated chemical system, and it certainly contains molecules antagonistic to sperm, even if, as Von Dungern's experiments indicate, it also contains some that are not.

10. INTERPRETATION OF SOME PHENOMENA OF NORMAL
FERTILIZATION IN NEREIS

To observe all the details of normal fertilization it is desirable to inseminate in a suspension of India ink which will define the transparent substances exuding from the egg on insemination. the following observations can then readily be made on mixing a drop of the eggs in the ink suspension with a drop of opalescent sperm suspension: Hundreds of spermatozoa become attached to each egg almost immediately; those in contact with the egg do not show much activity, but are usually definitely oriented radially; the spermatozoa external to these are in active movement. In about a minute a clear fluid begins to exude from the egg and surrounds all attached spermatozoa and involves the immediate neighbors. The first exudate is quite fluid for it flows around the spermatozoa and does not sweep them away, but the movements of all the spermatozoa within the exudate cease suddenly. The flow continues, and then most of the spermatozoa are swept away from contact with the egg, for the later exudate is gelatinous in consistency. However, a good many spermatozoa remain in contact with the egg for some time, but these are detached one by one as the flow of the jelly continues, until only one remains. Some of the supernumerary spermatozoa are not carried away until five or more minutes after insemination.

The immediate prevention of polyspermy in Nereis appears to be due to the paralyzing effect of the egg-exudate poured out in response to the stimulus of the first effective spermatozoon. Polyspermy could take place in Nereis only under two conditions, namely, (1) if two or more spermatozoa *simultaneously* give the stimulus to the egg that causes excretion of the agglutinin; for the condition of stimulus appears to be that the spermatozoon be securely anchored to the egg; and all spermatozoa not securely attached at the moment the egg begins to secrete are prevented from securing attachment by the resulting paralysis; (2) if the reaction of the egg be slow and therefore localized at first to the region of an effective spermatozoon, opportunity will be afforded for attachment of other spermatozoa.

mented with the spermatozoa of sea-urchins by the method of Pfeffer and came to the conclusion that "the spermatozoa of the Echinoidea are not attracted to the egg by means of any special substance excreted by the latter. The vast number of spermatozoa and the large size of the eggs are sufficient to ensure the necessary contact taking place." Von Dungern ('02) also rejects the idea of an egg-secretion attracting the spermatozoa in the case of sea-urchins and starfish. Morgan, Payne and Brown ('10) also accept Buller's interpretation, and there has recently been a tendency among biologists to reject chemotaxis of the spermatozoon as a factor in the fertilization of the egg.

Previous observers have worked either with Pfeffer's capillary tube method, or with the eggs themselves. I made a sufficient number of experiments with capillary tubes to convince myself that this method of experimentation is many times less effective than the method I employed. As illustration: July 4, 1912: I filled three pieces of capillary tubing with a concentrated solution of CO_2 in sea-water, with 10 per cent and 1 per cent of this solution and broke off short pieces—neither end of which had been in the solution—to be tested. These pieces were then introduced into an active sperm suspension of *Nereis* beneath a raised cover-slip. In the course of a few minutes a decided positive reaction was obtained with the first and second tubes, the sperm appeared to stream into the open mouth of the capillary tubes and soon formed white plugs at the mouths of the tubes. The tube containing 1 per cent CO_2 sea-water and a control tube with sea-water alone showed no reaction. In a repetition of this experiment the 1 per cent CO_2 sea-water and control were negative, and the 10 per cent showed only slight reaction. The diameter of the lumen of the tube was 0.48 mm. A much finer tube of 10 per cent CO_2 sea-water showed no reaction at all. With tubes of the size first used 10 per cent CO_2 sea-water is near the minimum for a positive reaction. But a drop of 0.5 per cent CO_2 sea-water injected into a similar suspension causes chemotactic response. Tubes of the size used are therefore about twenty times less effective as indicators of the chemotactic reaction, stated in terms of percentage of CO_2 required, than the injected drop method. It

is obvious that the size of the tube is a fundamental condition of the experiment, both because the diffusion is a factor of size and also because of the interference of thigmotactic reactions of the spermatozoa at the mouth of the tube with the purely chemotactic response. Buller does not state what was the size of the tubes that he used in his experiments. But, if the delicacy of the reaction was reduced to one-twentieth by the tube, his failure to get a positive reaction in tubes containing sea-water taken from over eggs is not surprising.

Von Dungern drew his conclusions from observing the behavior of spermatozoa mixed with eggs. Many embryologists, like myself, have made hundreds of observations of this kind; but it is obvious that the conditions thus created render an analysis of the behavior of the spermatozoa impossible. In some experiments I introduced a drop of eggs in sea-water into a sperm suspension beneath a raised cover, and obtained the typical ring formation of spermatozoa with reference to the group of eggs considered as a whole. But within the group any evidence of chemotactic reaction is clearly impossible.

As to the rôle that chemotaxis as a principle may play in the fertilization of the ova in nature it is difficult to form a clear conception. It may be little and it may be considerable. In the first place it may be noted that, although the echinids have been favorite subjects for research, but little appears to be actually known concerning their breeding behavior. In the second place we do not know the distance to which the secretion from an isolated egg will diffuse. But even if we assume that it extends effectively only a short distance in terms of the egg-diameter the result would be essentially to immensely increase the chances of scattered spermatozoa to become entangled in the jelly of the egg. Measurements of the effective radius of diffusion of the egg-secretion could, I believe, readily be made by the method employed in my work and the results of this might enable us to form some clearer idea of the possible significance of chemotaxis taken by itself in the meeting of the germ-cells.

The present results merely show that it may be a factor of some significance. The quickness and readiness of the reaction of

spermatozoa of *Arbacia* and *Nereis* to the secretion of their own kind of eggs is certainly surprising.

Another effect of their secretions that should be taken into account in this connection is the stimulating effect on the spermatozoa. This is more marked in some animals than in others. Thus the spermatozoa of *Nereis* are so active in the sea-water alone that but little effect of the egg-secretions can be noted; in the case of *Arbacia*, although the sperm are quite active in pure sea-water yet the egg-secretions greatly increase their activity for a brief time. In the case of the star-fish, according to Von Dungern's account, the spermatozoa tend to be very inactive in pure sea-water, but are aroused to intense activity by the secretions of the ova.

In different animals, therefore, we may expect to find some difference in the effect of egg-secretions on the activity of the spermatozoa. But the fact that in such widely separated forms as *Arbacia* and *Nereis* secretions of the egg cause strong positive chemotaxis of the spermatozoa inclines one to the view that such a reaction may be very wide spread. In a form in which egg-secretions are both activating and directing in their action, the importance of such secretions in favoring the preliminary steps in fertilization can hardly be doubted.

The experiments, like Pfeffer's earlier ones, indicate that the factor of specificity is probably subordinate in the purely chemotactic response. CO_2 and acids are in no sense specific, but they are very effective chemotactic agents with *Nereis* spermatozoa. But the case of *Arbacia* serves to indicate that substances of the egg, whether specific or not, are more generally effective than simple chemical substances, for it requires such substances, apparently, in the case of *Arbacia* to produce a reaction of the spermatozoa comparable in quickness and precision to the reaction of *Nereis* spermatozoa to acid, CO_2 , or the secretions of its own eggs.

We have seen that in some respects the chemotactic behavior of spermatozoa of *Nereis* and *Arbacia* is different depending on their relative sensitiveness to CO_2 and other agents. The much greater power of resistance of spermatozoa of *Chaetopterus* and

Loligo to CO_2 (see p: 528) indicates still greater differences in characteristic behavior. And it may be that the differences of chemotactic behavior of the spermatozoa of various animal phyla will turn out on investigation to be extensive. I am very far, therefore, from wishing to generalize any of the principles that we have found to hold true for *Nereis* and *Arbacia*. Sound generalizations must be based on much more extensive work. I have made some observations on the sperm of *Platynereis* megalops which demonstrate great differences as compared with *Nereis* in spite of the close relationship, correlated, no doubt, with differences in breeding behavior. While the common form of organization of flagellated spermatozoa points to fundamental principles of behavior in common, yet it must not be forgotten that each kind of spermatozoa has the chemical composition of the species, and may therefore have entirely specific forms of behavior.

The agglutination of the spermatozoa by normally formed egg-exudates of the same species indicates the possibility of studying the chemistry of fertilization directly through use of the spermatozoa as indicators. The very few and incomplete results which I was able to obtain in the time at my disposal seem to me to indicate a fruitful line of work. It would be interesting, for instance, to investigate whether or not the ova of hermaphrodite animals produce a sperm auto-agglutinin, that is, an agglutinating substance for spermatozoa of the same individual. Morgan's work on *Ciona* has shown that the failure to self-fertilize is in this case due to failure of penetration of the spermatozoon. It is difficult, as he points out, to explain this on any mechanical grounds. But if a specific agglutination is a necessary step in union of ovum and spermatozoon, the failure to produce an auto-agglutinin would explain the failure of self-fertilization. We would have in this event a precise parallel to the usual failure to produce blood auto-agglutinating substances in experiments on immunity, though iso-agglutinins are readily produced.

Godlewsky ('11) has shown that there is an antagonism between the sperm of certain animals (*Chaetopterus* and *Echinids*) which destroys the fertilizing power of each when mixed together for a certain length of time. He compares this to the antagonistic

action of heterogenous haemolytic sera on one another; and concludes that his results strongly confirm Loeb's theory that the spermatozoon initiates development by means of a lysin.

Without discussing the interpretation, and considering only Godlewsky's most interesting results, another parallel is furnished to immunity phenomena. We may confidently expect, therefore, that study of the reactions of spermatozoa will break a new path into the field of fertilization.

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THE EFFECT OF EXCRETION PRODUCTS OF INFUSORIA ON THE SAME AND ON DIFFERENT SPECIES, WITH SPECIAL REFERENCE TO THE PROTOZOAN SEQUENCE IN INFUSIONS

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It is well known that a hay infusion presents a kaleidoscopic series of phenomena from its inception until it finally reaches a stage of sterility, or, in the presence of sunlight, of practically stable equilibrium in which animals and green plants become so adjusted that a veritable microcosm exists. In an attempt to elucidate some of the complex factors involved in the faunal and floral changes of typical infusions a series of observations of a considerable number of infusions was made,¹ and the following conclusions, among others, were reached:

1. In hay infusions, seeded with representative forms of the chief groups of Protozoa, there is a definite sequence of appearance of the dominant types at the surface of the infusion, that is, Monad, Colpoda, Hypotrichida, Paramaecium, Vorticella and Amoeba.

2. The sequence of maximum numbers and of disappearance is identical with that of appearance, except that apparently the position of Amoeba advances successively from the last (sixth) place to the fifth place and then to the fourth place.

3. Emphasis is put upon the strictly biological interrelations (for example, those involving food and specific excretion products) of the various forms as the most important determining factors in the observed sequence.

The interdependence of the organisms of a hay infusion is so complex that, taken as a whole, it is quite beyond the possibility of analysis, and accordingly the logical method of approach to the subject was to study the effects of isolated organisms on themselves and on each other. The first essay in this direction con-

¹ L. L. Woodruff, Observations on the origin and sequence of the protozoan fauna of hay infusions. Jour. Exp. Zool., vol. 12, no. 2.

sisted of a study of the effects of media contaminated with the excretion products of a single form (*Paramecium*) on its rate of reproduction.² The results showed that paramaecia excrete substances which are toxic to themselves when present in their environment and reduce the division rate. This fact was interpreted as indicating that excretion products play an appreciable part in determining the period of maximum numbers, rate of decline, et cetera, of this animal in hay infusions.

In view of these results it was of interest to attempt to answer the following questions:

1. Are the excretion products of paramaecia specifically toxic to this species, or do they also influence the reproductive rate of other species?

2. Do other species in the observed sequence of forms of the infusion fauna produce excretion products which are specific, or otherwise, in their action?

3. Do species which succeed each other in the observed sequence produce substances which influence mutually their development and tenure of life in the infusion microcosm?

The present paper presents results which, it is believed, contribute to the solution of these questions with respect to *Paramecium* and two hypotrichous forms, *Stylonychia pustulata* and *Pleurotricha lanceolata*. The series of experiments described were made in an endeavor to determine the influence of media contaminated with the excretion products of paramaecia on the rate of reproduction of *Paramecium* and of the hypotrichous forms; and also to determine the influence of media contaminated with the excretion products of the hypotrichs on the rate of reproduction of these forms and of *Paramecium*.

It was decided to study the problem with respect to paramaecia and the hypotrichs because the latter have been shown, in the earlier paper, to occur in maximum numbers just before the dominance of the paramaecia, and because they lend themselves readily to such experimental methods as the present study involves.

² L. L. Woodruff, The effect of excretion products of *Paramecium* on its rate of reproduction. Jour. Exp. Zool., vol. 10, no. 4.

The paramaecia used in this work were from my pedigreed race of *Paramecium aurelia* (I). This was at the 2800th generation in January, 1912, when the study was started and had attained the 3450th generation by the conclusion of the experiments in December of the same year. For the hypotrichous forms which were employed I am indebted to Mr. George A. Baitsell who supplied them from his pedigreed races. Emphasis is placed on the fact that the animals which formed the subjects of the experiments had been under observation for considerable periods of time (in the case of the paramaecia, for five years) and consequently their rate of reproduction and the exact conditions to which they had been subjected before the experiments were known. Further, since the pedigreed races were each originally started with a single individual, all the specimens of the respective species were 'sister' cells and therefore all the experiments were made on the 'same protoplasm.'

A weak extract of hay was employed as a culture medium, which was made by boiling about 10 grams of hay in a liter of water for five minutes. The hay was then immediately filtered off and the liquid distributed equally in three flasks and allowed to cool. About a dozen paramaecia and a dozen hypotrichs were then isolated from their respective pedigreed cultures and allowed to mingle together in a watch glass of hay infusion. Three clean cover glasses were taken and on one was placed a drop of the infusion from the watch glass with a few of the paramaecia, on another was placed a drop with a few of the hypotrichs, and on the third was placed simply a drop of the infusion. One of these cover glass preparations was dropped into each of the three flasks of culture medium already prepared, which were accordingly designated *P*, *H* and *O* respectively, signifying paramaecia seeded, hypotrich seeded, and minus protozoa. The flasks were then allowed to stand at room temperature for from five to ten days, or until an enormous growth of the seeded forms had appeared, and then the media were ready to be studied. These three flasks thus contained the same culture medium and bacterial flora, and one differed from the other only in the presence of the protozoan with which it was seeded at the start.

The length of the individual experiments varied with the development of the organisms in the culture medium flasks. Obviously, it was necessary that there should be very heavy and essentially similar growths of both the paramaecia and the hypotrichs before the test of the media began, and frequently the medium had to be discarded and a new lot started, owing to the failure of heavy growths to develop. The more rapid rate of division of the hypotrichous forms rendered it expedient to seed the cultures with a few less individuals of these forms than of paramaecia in order to have the development of the two flasks synchronous. Again, the actual experiments could be continued only while heavy growths of the respective species persisted and consequently the length of the experiments varied, though a great majority comprised five days. In many cases it was found impossible to conduct series of paramaecia and hypotrichs simultaneously—but all data which are directly compared to determine the results, were run at the same time and consequently temperature changes are without influence.

The general conduct of the experiments was similar to that employed in my former work on the subject and therefore it is unnecessary to describe it in detail again. In each individual experiment four animals were isolated with the aid of a Zeiss binocular microscope and placed on separate depression slides and bred in the medium to be tested, which was supplied fresh daily during the period of the experiment. The average rate of division of these four lines, again averaged for the number of days of the experiment, afforded the data on which this paper is based.

The details of a couple of typical experiments will best illustrate the *modus operandi*. On January 18, 1912, sixteen paramaecia were isolated from the main pedigreed culture and each one was placed on a clean depression slide. Four of these were supplied with medium from flask *P* from which the paramaecia had been entirely removed,³ four were supplied with culture medium from flask *H* from which the hypotrichs had been care-

³ The infusoria were removed by filtering the medium through filter paper and then picking out with a pipet any of the animals which happened to pass through the paper. Medium *O* was also filtered in order to make the treatment the same for all the types of culture media.

fully eliminated, and eight (comprising two sets) were isolated in media from flask *O* which was uncontaminated with paramaecia or hypotrichs. At the same time sixteen *Stylonychia* were similarly isolated on separate slides and treated identically the same as the paramaecia just described. There were then in this experiment thirty-two lines of cells forming eight sets designated as follows:

- Po1 = paramaecia on uncontaminated medium
- Po2 = paramaecia on uncontaminated medium
- Pp = paramaecia on paramaecia contaminated medium
- Ph = paramaecia on hypotrich contaminated medium
- Ho1 = hypotrichs on uncontaminated medium
- Ho2 = hypotrichs on uncontaminated medium
- Hh = hypotrichs on hypotrich contaminated medium
- Hp = hypotrichs on paramaecia contaminated medium

This first experiment gave the following results which represent the average rate of division of the four lines of cells of each set, again averaged for the ten days during which they were continued:

- Po1 = 2.25 divisions per day
- Po2 = 2.31 divisions per day
- Pp = 1.95 divisions per day
- Ph = 2.46 divisions per day
- Ho1 = 4.50 divisions per day
- Ho2 = 4.45 divisions per day
- Hh = 4.15 divisions per day
- Hp = 3.95 divisions per day

This result is interpreted as showing that the *P* medium decreases the division rate while the *H* medium increases the division rate of Paramaecium. Further the *H* medium and the *P* medium decreases the division rate of the hypotrichs.

As the second example, Experiment 4 (February) may be cited. This was continued for four days with the following result:

- Po1 = 1.80 divisions per day
- Po2 = 2.05 divisions per day
- Pp = 1.35 divisions per day
- Ph = 1.95 divisions per day
- Ho1 = 3.73 divisions per day
- Ho2 = 3.75 divisions per day
- Hh = 3.40 divisions per day
- Hp = 3.50 divisions per day

These data indicate that the *P* medium reduces the division rate of both *Paramecium* and the hypotrichs, while the *H* medium reduces the rate of the hypotrichs and is without effect on that of the *paramecia*.

At the end of the four days, Po1 and Po2 were continued for four days more on the same medium while Pp and Ph were transferred to the *O* medium. Thus during this second phase of the experiment all lines were on the uncontaminated medium. The results were:

Po1	= 2.10 divisions per day
Po2	= 2.20 divisions per day
Ppo	= 2.10 divisions per day
Pho	= 2.00 divisions per day

From these figures it is apparent that placing the Pp on the *O* medium brought about in the Ppo series a division rate (within the limit of error) the same as that of the lines (Po1 and Po2) on the *O* medium from the start, thus proving that the reduction of the rate in the Pp series was a result of the *P* medium.

A comparison of the data from these two experiments shows that the results are not in entire agreement; that is, in Experiment 1 the *H* medium favored the division of *Paramecium*, while in Experiment 4 it was without influence. Such discrepancies are not surprising when the large number of factors involved in procuring heavy growth of the forms are taken into consideration, but they make it apparent that results of value cannot be secured without many repetitions of the experiment so that incidental disturbing factors can be eliminated. Accordingly, a series of experiments were made which involved the observation of more than 8000 individuals and the isolation of over 2000 animals. The results of the entire series may be best interpreted from the following brief table. In the first column (designated 'minus') the average division rate of the series was below that of the controls and beyond the limits of error as indicated by the differences between the two controls. In the second column (designated 'neutral') the average division rate was the same as the controls or within the limits of error as indicated by the differences between the two controls. In the third column (designated

'plus') the average division rate was above that of the controls and beyond the limits of error as indicated by the difference between the two controls.

	MINUS	NEUTRAL	PLUS
Pp.....	4	0	0
Hh.....	7	1	0
Ph.....	3	4	5
Hp.....	3	4	1

It is apparent from this table that the four experiments which repeat the work already published on *Paramecium* substantiate completely the conclusions there reached that paramaecia excrete substances which are toxic to themselves when present in their environment and inhibit their rate of reproduction. It is also evident that the hypotrichs excrete substances which inhibit their rate of reproduction since the division rate was reduced in seven out of the eight experiments—the exception indicating neutrality.

The results from the reciprocal action of the *P* and the *H* media are not so conclusive. Taken at their face value the figures would indicate that paramaecia excretion products are, on the whole, neutral or slightly inimical to the hypotrichous forms, while the latter's excretion products are, on the whole, slightly favorable to paramaecia.

Accepting for the moment this interpretation of the results, the data are, a priori, decidedly interesting. It having been shown in an earlier paper that, on the average, the hypotrich maximum at the surface of an infusion is passed before the corresponding period in the development of the paramaecia is attained, one would expect, if the hypotrichous species and the paramaecia mutually influence each others life in the infusion microcosm that the hypotrichs' products would produce a favorable medium for the approaching paramaecium maximum, while the products of the increasing hordes of paramaecia would render the medium unfavorable for the hypotrichs and so contribute to their decline. Obviously, however, the data secured are not sufficiently concordant to render this hypothesis secure though they trend in that

direction. It is perhaps too much to expect that experiments of this nature would show conclusively such a delicate adjustment as must exist between mutually associated forms in the inconceivably involved infusion complex.

These experiments, however, show conclusively, it is believed, that both paramaecia and hypotrichs produce substances which are specifically toxic to themselves. This result is made doubly secure by the very lack of concordance of the results secured from the study of their reciprocal action. The data show, for example, that media which have supported very heavy growths of hypotrichs are clearly inimical to the reproduction of the hypotrichida, while the same media are favorable or neutral to the development of *Paramaecium*. This rules out completely the possibility, discussed in the earlier paper,¹ that the depressing effects observed in this and the previously published experiments are the results of changes in the quantity or quality of the bacterial flora (on which the animals are dependent for food) in the protozoa seeded and protozoa free media.

SUMMARY

1. *Paramaecia* excrete substances which are toxic to themselves and these tend to inhibit the rate of reproduction.
2. *Hypotrichs* excrete substances which are toxic to themselves and these tend to inhibit the rate of reproduction.
3. These excretion products are essentially specific in their action since their presence does not uniformly influence the rate of reproduction of other species.
4. The data secured, therefore, emphasize the importance of specific excretion products as a factor in determining the limits of development of individual forms in the infusion microcosm but do not indicate that these specific products are of great importance in relation to the rate of development of associated species.

¹ Page 578.

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